Measurement of Intestinal Permeability in Surgical Patients

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Statement of Originality

This thesis has been prepared by the candidate. The experimental work described in this thesis was performed solely by the candidate, except where clearly stated (section 2.1). This thesis work has not been submitted for any other degree or professional qualification.

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Abstract

BACKGROUND: Intestinal permeability (IP) is often used as a surrogate marker of gut barrier function. Conventional methods of measuring intestinal permeability employ dual sugar probes. These undergo colonic fermentation and only permit assessment of small intestinal permeability. Using sucralose (a non-fermented sugar) a novel triple sugar test was developed, which measured both colonic and small intestinal permeability.

AIM: The aim of this study was to investigate the use of a triple sugar test of intestinal permeability as a surrogate marker of gut barrier function in surgical patients.

METHODS: Original laboratory work included the development of a technique for the quantification of urinary sucralose using high performance liquid chromatography (HPLC) with refractive index detection. Other techniques used included HPLC analysis of urinary lactulose and L-rhamnose, quantification of urinary ^{51}Cr-EDTA by gamma counting, and a lactulose-hydrogen breath test. The triple sugar test involved ingestion of a test drink containing sucralose (5g), lactulose (5g) and L-rhamnose (1g). Urine was collected for 24 hours in two aliquots (first five and last 19 hours) and sugar concentrations determined by HPLC. A ^{51}Cr-EDTA test was administered separately as an independent measure of "whole gut" permeability. Healthy volunteers (n=21) and ileostomists (n=18) were studied in order to investigate the sites of absorption of sugar probes. A number of patient groups were then studied; these included subjects with

Crohn's disease (n=16), acute colitis (n=18), irritable bowel syndrome (n=11), acute pancreatitis (n=9) and patients undergoing chemotherapy (n=7).

RESULTS: Assays for urinary sugars were both accurate and precise (coefficient of variation approximately 5%). Studies in ileostomists and controls indicated that 24-hour sucralose excretion represented "whole gut" permeability, whereas the five-hour lactulose/rhamnose excretion ratio represented small intestinal permeability. Small intestinal permeability was increased in subjects with Crohn's disease ($p=0.007$) and acute pancreatitis ($p=0.004$), *versus* controls. "Whole gut" permeability was significantly increased in patients with Crohn's ($p=0.001$) and pancreatitis ($p<0.001$), and significantly reduced in patients undergoing chemotherapy ($p=0.012$). The proportion of sucralose excreted in the last 19 hours of collection was significantly increased in patients with Crohn's ($p=0.026$), acute colitis (0.023) and acute pancreatitis ($p=0.049$), implying an increase in colonic permeability.

CONCLUSIONS: The triple sugar test enabled simultaneous quantification of small intestinal and "whole gut" permeability. By examining temporal probe excretion rates, isolated colonic damage could be identified. Sensitivity, however, was relatively low. The finding of increased colonic permeability in patients with acute pancreatitis provides a rationale for the use of colon-specific therapies in this condition.

Part 1: Introduction

1.1. Overview

Recent years have seen an increasing recognition of the fact that the gastrointestinal tract has functions other than simply the digestion and excretion of foodstuffs. The gut is also a metabolic and immunological organ that serves as a barrier against living organisms and antigens within its lumen. "Gut barrier function" is the ability of the gastrointestinal tract to prevent the passage of intraluminal pathogens to extraluminal sites. In the "gut origin of sepsis" hypothesis, a breakdown in gut barrier function is postulated to result in the trans-epithelial migration of bacteria and endotoxin, which then contribute to the development of systemic inflammation, distant sepsis, and multiple organ failure (Saadia *et al*, 1990, Swank *et al*, 1996).

One of the difficulties encountered by investigators studying the "gut origin of sepsis" hypothesis in humans is that gut barrier function is exceptionally difficult to measure. In contrast to animal studies, it is rare to have access to portal blood, mesenteric lymph or perfused intestinal tissue in clinical studies. One non-invasive method of investigating the gut is to measure intestinal permeability. This involves the administration of an oral test solution followed by urine collection, and is practical in the majority of surgical patients. Tests of intestinal permeability measure passive paracellular trans-epithelial diffusion of probe molecules. It is hypothesised that this is related to epithelial integrity and gut barrier function. Until recently, tests of intestinal permeability in common usage have only investigated the small intestine (disaccharide/monosaccharide tests). In this

study, a new "triple sugar" technique of assessing permeability of both the small intestine and colon is investigated.

1.2. Aim

The aim of this study was to investigate the use of a triple sugar test of intestinal permeability as a surrogate marker of gut barrier function in surgical patients. It was hypothesized that the use of a non-fermented sugar (sucralose), in combination with a fermented sugar (lactulose) would allow quantification of small and large intestinal permeability separately. This might enable site-specific investigation of gut barrier function in patients with primary gastrointestinal disease and in whom secondary gut insult was suspected.

1.3. The Gut Barrier and the Gut Origin of Sepsis Hypothesis

1.3.1. The Gut Barrier

Components of the gut barrier include gastrointestinal microflora, passive defence mechanisms such as gastric acid, bile salts, digestive enzymes, peristalsis and the intestinal mucous layer, immunological defence mechanisms such as macrophages and the gut-associated lymphoid tissue (GALT) and the intestinal epithelium itself. Intestinal permeability testing is primarily a measure of epithelial integrity. This aspect of gut barrier function is discussed in greater detail below.

1.3.1.1. Epithelial Micro-Anatomy

The intestinal epithelium represents the major structural component of the gut barrier. It is comprised of a single layer of columnar epithelial cells resting on a basement membrane. Epithelial cells are connected to one another by junctional complexes. These complexes were first characterised in rodents by Farquhar and Palade in 1963, who found striking similarities in the microanatomy of the intercellular region of epithelia taken from such diverse sites as the GI tract, exocrine glands, hepatic ducts and the renal nephron (Farquhar *et al*, 1963).

Using electron microscopy, Farquhar and Palade demonstrated that junctional complexes are formed by three structures; the zonula occludens (tight junction), zonula adherens (intermediary junction) and macula adherens (desmosome). The tight junction (TJ) is situated on the luminal side of the complex and is shaped like a narrow belt which

encircles epithelial cells at their apical pole. At the TJ the lateral membranes of adjacent cells are closely apposed, the gap between cells at this point being of the order of 20nm. On electron microscopy the TJ appears to be traversed by strands, the density of which varies between different types of epithelium. The zonula adherens consists of an intercellular space occupied by low-density material, and the desmosome is a discontinuous "button-like" disc of dense material, apparently acting as an intercellular attatchment device (Farquhar *et al*, 1963).

1.3.1.2. Physiology

Whilst highly developed for the absorption of nutrients, exclusion of pathogens and numerous other functions, the intestinal epithelium remains fundamentally a biological membrane. In terms of water and solute movement it behaves in a similar manner to tissues as diverse as capillary endothelium and frogskin. In the second half of last century a number of advances were made by membrane physiologists, many of which have aided the understanding of water and solute movement across the intestinal epithelium.

The first of these was the introduction of the "pore theory of permeability" by Pappenheimer and others. This theory was developed following the observation of water and solute movement across capillary endothelium. In its simplest form it supposes that biological membranes are "pierced with numerous ultramicroscopic openings which are in general too small to allow the passage of plasma protein molecules," but which will

allow "passage of water and non-protein constituents of the plasma" (Pappenheimer *et al*, 1951).

The second important advance was the discovery that a significant proportion of ion flux across biological membranes occurred between, rather than across epithelial cells. In the 1960's and 70's, microelectrode technology allowed measurement of potential differences at various sites across epithelial surfaces, enabling calculation of trans-epithelial electrical resistance (TEER). It was found that resistance to ion flow was much lower across the intercellular regions than across epithelial cell bodies. This paracellular "shunt pathway" was not an inert epithelial property, but appeared to be regulated in some manner. For instance, frogskin permeability increased greatly at certain times of the year, or when the tissue was placed in hyperosmolar solution (Fromter *et al*, 1972, Ussing *et al*, 1964).

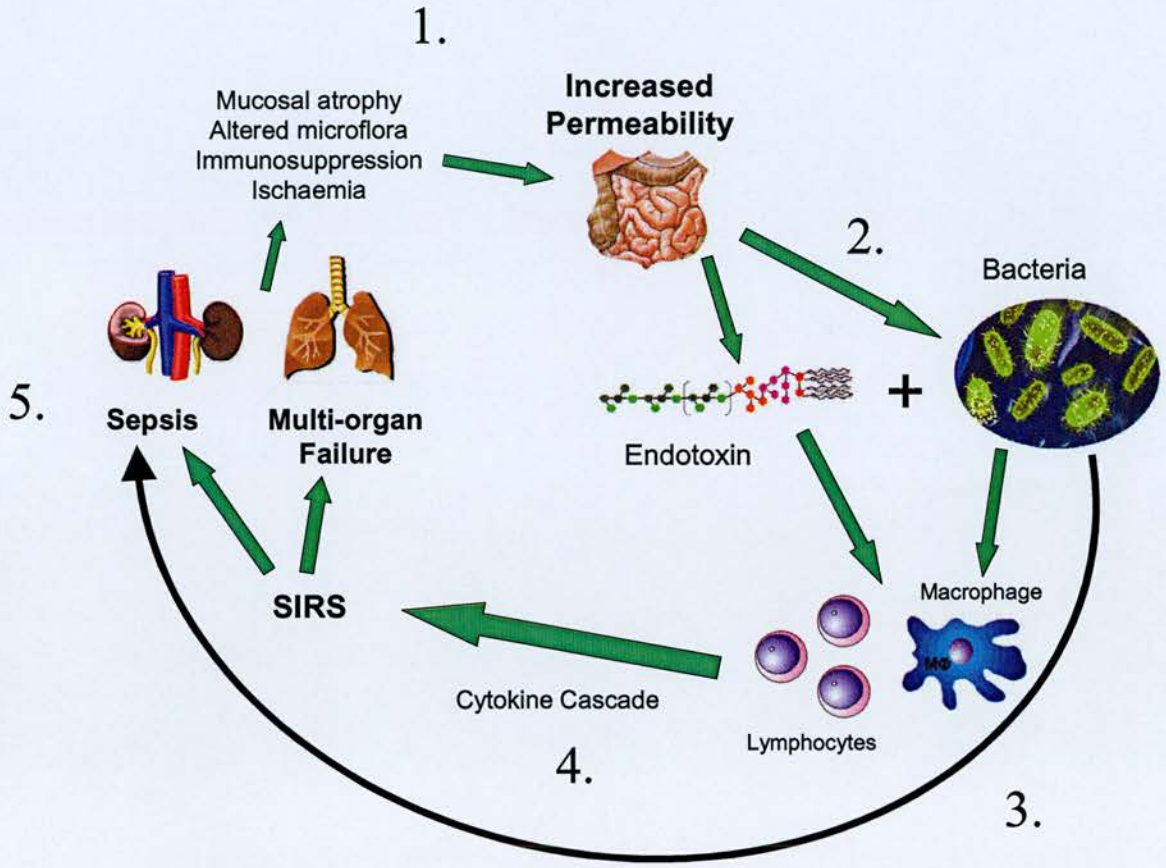
From the work of these physiologists and others it can be concluded that the intestinal epithelium behaves like a membrane pierced by multiple pores, many of which are situated in the intra- or paracellular region. The third important advance was the realisation that these paracellular pores are controlled by tight junctions. In experimental models, permeability characteristics correlate well with electron-microscopic and freeze-fracture analysis of tight junction composition (Madara *et al*, 1982, Marcial *et al*, 1984). Far from being the impermeable gasket they first appeared, tight junctions show a high degree of plasticity and appear to regulate paracellular ion flux (Madara, 1989). Whilst

under normal conditions larger molecules are not admitted (Farquhar *et al*, 1963), this is not a universal finding. After cholinergic stimulation, molecules of up to 40,000 daltons have been demonstrated to pass through the tight junctions of rat ileal epithelium (Phillips *et al*, 1987).

1.3.2. The Gut Origin of Sepsis Hypothesis

This thesis is based upon the assumption that changes in intestinal permeability reflect changes in gut barrier function. In the "gut origin of sepsis" hypothesis, it is postulated that intestinal insult caused by a variety of factors leads to a breakdown in gut barrier function. This enables intraluminal bacteria, bacterial products and ingested antigen to cross the intestinal mucosa and initiate local inflammation and systemic infection. The passage of bacteria across the intestinal wall in this manner is termed "bacterial translocation" (O'Boyle *et al*, 1998). The main events thought to occur in the "gut origin of sepsis" hypothesis are depicted in Figure 1.

Figure 1. The "gut origin of sepsis" hypothesis



Key

(1). Gut insult such as mucosal atrophy, altered microflora, immunosuppression or ischaemia results in damage to the mucosa and increased permeability.

(2). This allows bacteria and endotoxin to translocate across the mucosa into the submucosa and reach the gut associated lymphoid tissues (GALT).

(3). Interaction with macrophages and other immune cells results in the release of inflammatory mediators.

(4). Inflammatory mediators initiate a cytokine cascade which, if uncontrolled, can lead to the systemic inflammatory response syndrome (SIRS).

(5). The combination of uncontrolled systemic inflammation and translocating bacteria reaching the systemic circulation results in end-organ damage.

It is well known that systemic sepsis and multiple organ dysfunction syndrome (MODS) can be the result of an intra-abdominal or other septic focus (Polk *et al*, 1977). In a large proportion of patients, however, no septic focus can be identified, even when post-mortem data are available (Saadia *et al*, 1990). In these patients there is accumulating evidence to suggest that the gut plays a central role in the development of sepsis, the systemic inflammatory response syndrome (SIRS) and ultimately multiple organ failure.

The commonest bacterial isolates from critically ill patients are gut commensals such as *E. coli*, *Enterococci* and *Pseudomonas*. Whilst these organisms are commonly cultured from such sites as lungs, urine, wounds and blood, the most likely site of origin is the gastrointestinal tract (Emori *et al*, 1993, Marshall *et al*, 1993, Richardson *et al*, 1982). Patients in an intensive care unit (ICU) in whom there is evidence of bacterial colonisation of the upper gastrointestinal tract are more likely to suffer subsequent septic complications than those with sterile gastric aspirates (Marshall *et al*, 1988). In one study of 41 surgical ICU patients, gastric colonisation with *Pseudomonas*, *Staph. epidermidis* or *Candida* was associated with the development of invasive infection with the same organism in up to 90% of patients (Marshall *et al*, 1993). In a study of 279 general surgical patients, the presence of multiple organisms in a preoperative nasogastric aspirate was associated with a subsequent sepsis rate of 50%, compared with 15.3% in patients whose nasogastric aspirate was sterile ($p<0.01$) (MacFie *et al*, 1999).

To date, it has been possible to demonstrate bacterial translocation in humans only in patients undergoing abdominal surgery, when mesenteric lymph nodes can be harvested

for microbiological analysis. In this group of patients a number of factors are associated with an increase in translocation, including emergency surgery and intestinal obstruction (Brathwaite *et al*, 1993, Deitch, 1989, MacFie, 1997). In a series of 448 patients undergoing laparotomy those with evidence of bacterial translocation (15.4% of all patients) had a significantly higher rate of post-operative sepsis (45% sepsis rate) than those with sterile lymph nodes (19% sepsis rate) (O'Boyle *et al*, 1998).

There is, therefore, a growing body of evidence in support of the "gut origin of sepsis" hypothesis in a variety of patient groups. Most of the evidence in humans is circumstantial and relates to an association between the isolation of bacteria in nasogastric aspirates or mesenteric lymph nodes and the subsequent development of sepsis. In animal models there is more direct evidence demonstrating that critical illness results in gut barrier dysfunction and translocation of potentially pathogenic bacteria (Alexander *et al*, 1990, De Oca *et al*, 1993, Deitch, 1993, Kelly *et al*, 1997, O'Brien *et al*, 2002).

In order to transgress the intestinal epithelial barrier bacteria, endotoxin or any other intraluminal substance must pass either through enterocytes or between them. These two potential routes of trans-epithelial migration are termed the transcellular and paracellular pathways. The route taken by translocating bacteria is as yet uncertain. To date it has not been possible to directly visualise the process of bacterial translocation *in vivo*. In one experiment a mixture of *E. coli, C. albicans* and endotoxin was inoculated into the lumen of viable perfused intestinal loops in anaesthetised rodents (Alexander *et al*, 1990).

A 50% burn was then administered. Post-mortem examination of the bowel using electron microscopy demonstrated *Candida* and *E. coli* within enterocytes as early as one hour following thermal injury. Organisms were seen disrupting the microvilli on the luminal brush border, and extruding through the basement membrane on the other side of the enterocyte. Neither *E. coli* nor *Candida* was seen within the paracellular space. Organisms were identified in the lamina propria and lymphatics; most of these had been engulfed by phagocytic cells. These data suggest that the transcellular route is involved in bacterial translocation.

The mechanism by which bacteria translocate is likely, however, to be more complex than simple endocytosis by enterocytes. *In-vitro* studies using the HT-29 enterocyte cell line have demonstrated increased internalisation of enteric bacteria under conditions of increased *paracellular* permeability, such as incubation in calcium free solutions or with bacterial endotoxin. Under these conditions the enterocytes became rounded and bacteria were seen to adhere preferentially to the exposed basolateral cell membranes. On reversal of the experimental conditions there was restoration of normal cell shape and paracellular permeability, and bacterial internalisation returned to baseline levels (Wells *et al*, 1995, Wells *et al*, 1996). These data suggest that both trans- and paracellular pathways might be involved in the process of bacterial translocation.

1.4. Intestinal Permeability Testing

The investigation of intestinal permeability has a history which spans several centuries. Martin Lister and William Musgrave, in 1673 and 1701, published the results of experiments in which test solutions containing indigo ink were perfused into the intestinal lumen of dogs, and the lacteals subsequently examined for staining (Lister, 1673, Musgrave, 1701). Like much subsequent work on intestinal permeability, the results of the two similar studies were conflicting, Lister found that the lacteals remained "ever white and uniform," whilst Musgrave describes discoloration of the lacteals which, when cut "afforded a blue liquor running forth on the mesentry."

1.4.1. Physiology

Intestinal permeability is that property of the intestinal epithelium which allows molecules to pass through by non-mediated diffusion (Travis *et al*, 1992). Intestinal permeability should be distinguished from membrane permeability, which relates to permeation of molecules of molecular mass less than 150 Da, such as sodium and chloride ions (Schultz *et al*, 1961, Travis *et al*, 1992). In this thesis it has been assumed that "intestinal permeability" refers to the unmediated passage of water-soluble compounds across the intestinal epithelium (Meddings, 1997).

1.4.1.1. The Use of Oral Test Probes

The basic premise of modern tests of intestinal permeability is that the urinary excretion of an orally administered test substance ("probe") relates to the permeation of that probe across the intestinal epithelium, and hence to intestinal permeability. Many substances have been used as permeability probes, including monosaccharides, oligosaccharides, ^{51}chromium-labelled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) and polyethylene glycol (PEG). An "ideal" marker of intestinal permeability should be biochemically inert, insoluble in lipids (lipid-soluble molecules can pass directly through cell membranes, confounding the investigation of aqueous pore permeation), and should cross the intestinal epithelium by non-mediated diffusion via well-defined pathways. In addition it should undergo 100% renal excretion following intravenous injection (Travis *et al*, 1992). Unfortunately no probe molecule in current use fulfils all of these criteria. In particular, there remains controversy about the routes taken by probe molecules as they cross the intestinal epithelium.

1.4.1.2. Routes of Probe Permeation

Hydrophilic molecules have much slower rates of absorption than lipid-soluble molecules of similar size, and the way in which they are absorbed implicates non-mediated diffusion as the transfer mechanism (Hober *et al*, 1937, Menzies, 1974). With the exception of PEG, permeation is restricted with increasing molecular weight (Hamilton *et al*, 1987, Hober *et al*, 1937, Maxton *et al*, 1986). Probe cross-sectional diameter, determined using complex computer modelling, shows an even stronger negative correlation with permeability (Hollander *et al*, 1988). These data are consistent with Hober's postulation

in the 1930's that "absorption of these substances is like a diffusion through a sieve-like membrane, the maximal pore diameter of which controls the limiting molecular volume allowing permeation" (Hober *et al*, 1937).

Despite over 30 years of extensive research into gastrointestinal permeability, the exact location of these pores in the intestinal epithelium remains uncertain. Additional questions remain over the number of discreet pore populations. Hydrophilic probes with molecular weight of greater than 180Da (such as oligosaccharides and ^{51}Cr-EDTA) permeate at a rate of approximately 0.5% over six hours in normal individuals. They exhibit a marked increase in permeation when administered in hyperosmolar solution, in combination with cetrimide, and in the presence of intestinal inflammatory disorders such as coeliac and Crohn's disease (Table 2). These "large" probes have been shown to be unable to penetrate erythrocytes (Menzies, 1984). In contrast, smaller probes such as the monosaccharides mannitol and L-rhamnose permeate at rates of 8-20% over six hours in healthy controls, show little change in response to hyperosmolar stress or cetrimide, and exhibit reduced permeation in coeliac disease. Unlike lactulose, L-rhamnose has been shown to enter erythrocytes (Menzies, 1984). These differences in permeation between "small" (<180 Da) and "large" (>180 Da) probes imply that they are absorbed via separate pathways. Theories regarding the anatomical location of these pathways are discussed below.

Trans vs. Paracellular Pore Theory

The most popular theory regarding the routes taken by probe molecules as they cross the intestinal epithelium is that proposed by Menzies (Menzies, 1984). In this theory of "para- *versus* trans-cellular" permeation it is proposed that there are two distinct pore populations; an abundant population of small (0.4-0.7nm radius) pores, through which monosaccharides pass, and a small population of large pores (6.5nm radius), through which the larger probes (along with a proportion of the smaller probes) pass (Travis *et al*, 1992). It is proposed that the larger pores are located in the paracellular space and comprise the tight junctions, whilst the small pores exist on the brush border, within the lipid membrane of the enterocytes. Accordingly, urinary excretion of oligosaccharides or ^{51}Cr-EDTA reflects paracellular permeability, whilst urinary excretion of monosaccharides reflects primarily transcellular permeability.

The structure and function of tight junctions has already been discussed (section 1.3.1). Tight junctions account for less than 5% of the total surface area of the intestinal epithelium (Marcial *et al*, 1984), which is compatible with the theory that these form the population of large pores that admit oligosaccharides and ^{51}CrEDTA.

After traversing the epithelium via trans- or paracellular routes, probes must cross the basement membrane, extracellular matrix and capillary or lymphatic endothelium in order to enter the systemic circulation. This "common pathway" does not appear to regulate the movement of molecules smaller than proteins, and so does not represent a rate-limiting step in the permeation of marker molecules (Travis *et al*, 1992).

Other Theories

Whilst most authors accept that the paracellular route plays an important role in the permeation of probe molecules, the importance, or even existence, of a transcellular route is controversial. Unlike tight junctions, which are clearly demonstrable by electron microscopy, pores in the apical enterocyte membrane have never been visualized. As a result of this failure to demonstrate transcellular probe flux, other theories have been developed to explain the differences in absorption between "large" and "small" probes.

In 1995, Bjarnason described a "single paracellular permeation" model (Bjarnason *et al*, 1995). In reviewing the work of Hollander and others, he hypothesised that the permeation of all probe molecules (including PEG) is inversely related to their molecular diameter, as calculated using computer modeling techniques. The different rates of probe permeation in health could therefore be accounted for by a single population of paracellular pores which varied in size, with a relative abundance of smaller pores (allowing permeation of PEG 400 and monosaccharides), and a far smaller population of larger pores (allowing permeation of all probe molecules). In order to explain the difference in lactulose and monosaccharide permeation seen in coeliac disease, he proposed that the larger pores are situated in the villus crypts, and the smaller ones at the villus tips. Thus, a disease which resulted in villus tip atrophy would lead to a drastic decrease in the number of small pores, resulting in a decrease in the permeation of "small" probe molecules (monosaccharides, PEG 400). Inflammation in the crypts might result in loosening of tight junctions, with resultant increased permeation of larger probe molecules. This would explain the increase in lactulose and ^{51}Cr-EDTA permeation seen

in coeliac disease. Although permeation of monosaccharides and PEG 400 would also increase in the crypts, there would be a net decrease, due to the more dramatic effect of villus tip atrophy. The decrease in pore size along the crypt-villus axis might be due to paracellular tight-junctions becoming "tighter" as enterocytes mature and migrate from the crypt to the villus tip. There is some experimental evidence in support of this hypothesis (Marcial *et al*, 1984).

A further proposal regarding the mechanism of probe permeation is the theory of vascular counter-current multiplication described by Bijlsma *et al* (Bijlsma *et al*, 2002, Bijlsma *et al*, 1995). The authors measured *in-vivo* intestinal permeability to lactulose and mannitol in humans and a variety of animal species. They also measured permeability *in-vitro*, using intestinal biopsy specimens mounted in Ussing-type diffusion chambers. They found that *in-vitro* lactulose/mannitol permeability ratios were similar across all species, being of the order of 0.8. However, permeability *in-vivo* differed between different species, with lactulose/mannitol excretion ratios ranging from approximately 0.6 in guinea pigs and rats, to approximately 0.03 in cats and humans. The difference was largely due to high levels of mannitol permeation in cats and humans *in-vivo*, which was not seen in other species, or when the intestinal epithelium was excised and mounted in diffusion chambers. This implied that the inter-species differences in intestinal permeability seen *in-vivo* were due to differences in gut function or architecture rather than epithelial structure. The authors proposed that the high mannitol absorption seen in humans and cats was due to hyperosmolar "drag" at the villus tip. Hyperosmolarity in

the relatively long villi of these species was postulated to develop as a result of a countercurrent multiplication system, the basis of which was the opposing flow of blood in villus capillaries and arterioles.

Summary

It can be seen from these diverse theories that intestinal permeability to orally administered test probes is not fully understood. In particular, the exact routes taken by probe molecules as they traverse the intestinal epithelium *in-vivo* remain uncertain.. It can also be seen that *in-vitro* tests of permeability often bear very little relation to events *in-vivo*.

One factor which is common to all theories is that larger molecules such as lactulose and ^{51}Cr-EDTA appear to permeate across tight juctions via the paracellular pathway. In the light of this, it can be concluded that the urinary excretion of these larger probes reflects tight junction integrity of the intestinal epithelium.

1.4.2. Test Probes

Over the last 30 years, there has been extensive use of non-invasive tests of intestinal permeability in humans. Many substances have been used as permeability probes, those discussed below include oligosaccharides (eg lactulose), monosaccharides (eg L-rhamnose, mannitol), ^{51}chromium labelled ethylenediaminetetraacetic acid (^{51}Cr EDTA) and polyethylene glycol (PEG).

1.4.2.1. Lactulose

Lactulose is a disaccharide of molecular weight 342.3 Da (Budavari *et al*, 1989). It is not hydrolysed by intestinal enzymes (Bjarnason *et al*, 1995), and undergoes almost 100% urinary excretion over 24 hours when administered intravenously (Elia *et al*, 1987, Maxton *et al*, 1986, Wheeler *et al*, 1978). These properties make lactulose a useful probe for the investigation of intestinal permeability. However, lactulose is a substrate for fermentation by colonic bacteria and is degraded on incubation with stool (Meddings *et al*, 1998). This has resulted in many authors stating that lactulose is not absorbed in the colon.

Jenkins *et al* demonstrated 24-hour urinary lactulose excretion to be 0.46% in six patients with ileostomies, compared with 0.5% in healthy controls (Jenkins *et al*, 1991). In a similar study, 24-hour lactulose excretion was 0.49% in three patients with ileostomies, compared with 0.36% in 22 healthy controls (Elia *et al*, 1987). These limited data also suggest that colonic absorption of lactulose is insignificant. Additional evidence for this

premise comes from data on the temporal excretion of lactulose. Maxton *et al* demonstrated that 73% of the total lactulose excreted over 24 hours was collected in the first five hours (Maxton *et al*, 1986). Elia *et al* demonstrated an almost identical figure of 72% in the first six hours (22 healthy volunteers) (Elia *et al*, 1987). Whole gut transit time, as measured by the appearance of blue dye in the stool, has been demonstrated to be greater than 24 hours in 11 of 12 healthy volunteers (Jenkins *et al*, 1991). Thus the fact that only 30% of 24-hour lactulose excretion occurred during the last 18 hours of collection also suggests that lactulose is degraded upon entering the colon. In the light of these data, lactulose is used as a measure of small intestinal permeability. It is of note, however, that the capability of the colon to absorb lactulose has not been directly assessed in humans.

Urinary Excretion in Healthy Volunteers

When using lactulose as a measure of small intestinal permeability, the standard urinary collection time following oral administration is five or six hours. Administration to healthy volunteers in concentrations approximately isotonic to plasma results in about 0.25% of the oral dose being excreted in the urine over this time period. Examples of lactulose excretion data taken from a number of clinical studies using healthy volunteers are shown in Table 1, along with excretion rates of co-administered monosaccharides (see below).

Table 1. Lactulose and monosaccharide excretion in healthy adult volunteers

Authors	No. of Subjects	Dose Administered	Time of Collection	Solution	Urinary Excretion (%)	Ratio of disaccharide to monosaccharide
Laker 1977	21	Lactulose 10g	5 hours	Iso-osmolar	0.3 (med)	_
Ukabam 1983	16	Lactulose 10g Mannitol 0.5g	6 hours	_	0.13 SEM 0.01 15.6 SEM 0.9	0.008
Ukabam 1984	25	Lactulose 10g Mannitol 0.5g	6 hours	(100ml)	0.15 SEM 0.01 15.9 SEM 0.88	_
Elia 1987	22	Lactulose 10g Mannitol 5g	6 hours	1300mosmol/L	0.26 SEM. 022 13.3 SEM 0.99	0.021 SEM 0.003
Juby 1989	12	Lactulose 5g Mannitol 2g	5 hours	1500mosmol	0.44 SEM 0.076 28.5 SEM 2.81	0.016 SEM 0.002
Fleming 1990	18	Lactulose 10g Mannitol 5g	6 hours	1350 mosmol/kg	0.33 SEM 0.04 11.9 SEM 0.84	0.027 SEM 0.003
Menzies 1990	10	Lactulose 5g Rhamnose 1g	5 hours	300mosm/L	0.3 SEM 0.03 14.7 SEM 1.8	0.023 SEM 0.003
Jenkins 1991	12	Lactulose 5g	24 hours	"iso-osmolar"	0.5 SEM 0.08	_
Teahon 1992	25	Lactulose 5g Rhamnose 1g	5 hours	1500mosmol/l	0.37 SD 0.21 9.4 SD 2.6	0.039 SD 0.02

(Continued)

Authors	No. of Subjects	Dose Administered	Time of Collection	Solution	Urinary Excretion (%)	Ratio of disaccharide to monosaccharide
Koltun 1993	10	Lactulose 5g Mannitol 1g	5 hours	100ml (<250mosm/L)	_	0.0017 SEM 0.0004
Roumen 1993	7	Lactulose 10g Mannitol 5g	6 hours	60ml	0.22 SD 0.09 21.9 SD 8.3	0.012 SD 0.005
May 1993	31	Lactulose 5g Mannitol 2g	5 hours	"iso-osmotic"	0.15 SEM 0.01 17.5 SEM 1.0	0.009 SEM 0.001
Wyatt 1993	30	Lactulose 10g Mannitol 5g	5 hours	1300mosmol/l	0.22 SEM 0.02 12.7 SEM 1.18	_
Pape 1994	6	Lactulose 10g Mannitol 5g	6 hours	50ml	_	0.56 SEM 0.24
Obinna 1995	8	Lactulose 10g Mannitol 5g	6 hours	50ml = 1350mmol/kg	0.25 SEM 0.04 19.6 SEM 7.01	0.013 SEM 0.0008
Johnston 1996	20	Lactulose 5g Rhamnose 1g	5 hours	250mosm/L	0.32 SD 0.1 13.4 SD 2.9	0.03 SD 0.01
Smecuol 2001	19	Lactulose 5g Mannitol 2g	24 hours	1800mosmol/L	_	Med 0.022, CI95 0.017-0.026

Numbers represent means unless otherwise stated
SEM: Standard error of the mean
SD: Standard deviation
Med: Median

Quantification of Urinary Lactulose

A variety of techniques have been used to quantify urinary lactulose and other sugars used in tests of intestinal permeability. Common analytical methods include quantitative thin-layer chromatography (TLC) (Menzies, 1973, Menzies *et al*, 1978), enzymatic analysis (Behrens *et al*, 1983), gas-liquid chromatography (GLC) (Laker, 1979) and high performance liquid chromatography (HPLC) (Delahunty *et al*, 1986, Sorensen *et al*, 1993). All of these methods are time consuming and exacting, and the choice of method is often based on available facilities and local experience. TLC and enzymatic techniques require separate estimation of mono- and disaccharides, whilst GLC requires time-consuming prior derivatisation. High performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) has been shown to allow relatively rapid simultaneous quantification of di- and monosaccharides in urine and plasma (Fleming *et al*, 1996, Fleming *et al*, 1993, Sorensen *et al*, 1993). Analytical recovery (mean ± SD) of urinary lactulose using this method in two studies was 97.6 ± 6.8% at a concentration of 6mg/L (Fleming *et al*, 1993) and 100 ± 6.1% at a concentration of 2mg/L (Sorensen *et al*, 1993). This technique was used for the quantification of urinary lactulose and L-rhamnose in the current study. Further details are given in section 2.3.1.

1.4.2.2. Dual Sugar Techniques

The urinary excretion of any orally administered probe will be affected by a number of factors in addition to the permeability of the intestinal epithelium. Premucosal factors include completeness of ingestion, gastric dilution and emptying, intraluminal dilution, intestinal transit time, integrity of the unstirred water layer and bacterial degradation. Postmucosal factors include intestinal blood-flow, metabolism, endogenous sugar production, tissue distribution, renal function, and completeness of urinary collection (Bjarnason *et al*, 1995, Travis *et al*, 1992).

In order to obtain a meaningful estimate of mucosal permeation from urinary excretion rates, an attempt must be made to control for this multitude of pre-and post-mucosal factors. This is commonly achieved by the use of a second probe marker ("control probe"), the final permeability data being presented as the ratio of [urinary excretion test probe] to [urinary excretion control probe]. Ideally, such a control probe would be affected by pre- and post-mucosal factors in an identical manner to the test probe, but cross the intestinal mucosa by a different route.

Over 20 years ago it was recognised that monosaccharides such as mannitol (MW 182.2 Da (Budavari *et al*, 1989)) and L-rhamnose (MW 182.2 Da (Budavari *et al*, 1989)) exhibited different permeability characteristics to disaccharides such as lactulose. The differing behaviour of di- and monosaccharide probes in coeliac disease, and after hyperosmolar and cetrimide stress is discussed above (section 1.4.1.2). As they appear to be absorbed by different routes, monosaccharides can be administered along with

lactulose, as "control" probes. Commonly used monosaccharides include mannitol and L-rhamnose. Expressing permeability as a ratio of disaccharide to monosaccharide excretion effectively negates the effect of pre and postmucosal factors on the final permeability result. Examples of disaccharide/monosaccharide excretion ratios in healthy volunteers can be seen in Table 1. Early studies in coeliac disease demonstrated that the use of dual sugar techniques increased the sensitivity of permeability testing, compared to the use of lactulose alone.

One notable exception to the rule that di- and monosaccharides are equally affected by pre and postmucosal factors is that of the renal clearance of L-rhamnose. Whilst 24-hour urinary recovery of parenterally administered lactulose and mannitol is close to 100% of the administered dose, the corresponding figure for L-rhamnose is only approximately 70% (Elia *et al*, 1987, Maxton *et al*, 1986). In calculating lactulose/rhamnose (L/R) ratios, some authors have advocated using a "corrected" L-rhamnose excretion rate, taking account of this "systemic loss" (Maxton *et al*, 1986), whilst others use absolute L-rhamnose excretion (Bjarnason *et al*, 1991, Johnston *et al*, 1996). For the sake of simplicity, absolute L-rhamnose excretion has been used in the current study.

1.4.2.3. Polyethylene glycol

Polyethylene glycol (PEG) polymers are available in a number of different molecular weights, and have the formula $H(OCH_2CH_2)_nOH$ (Bjarnason *et al*, 1995). They are not degraded by gastrointestinal flora, and have been used as markers of intestinal permeability following oral and intra-intestinal administration. The most commonly used is "PEG 400." This comprises a mixture of eight or more PEG polymers with molecular weights ranging from 194 to 502 Daltons (Maxton *et al*, 1986). Quantification in urine is usually performed using HPLC or GLC. With these techniques it is possible to separate out the various PEG polymers and quantify their permeation across the intestine separately (Delahunty *et al*, 1986, Sundqvist *et al*, 1980). First introduced to the field of intestinal permeability testing by Chatwick *et al* in 1977 (Chadwick *et al*, 1977), polyethylene glycol has been the subject of some controversy over the last two decades.

With an average molecular weight of 400 Da, it might be assumed that PEG would behave in a similar manner to lactulose (MW 342) and ^{51}Cr-EDTA (MW 340). Given that PEG is not a substrate for bacterial fermentation, urinary PEG excretion would thus represent whole-intestinal paracellular permeability. However, PEG differs from other probe markers of similar size in a number of important respects.

When administered orally to healthy volunteers, approximately 20% of ingested PEG 400 can be quantified in the urine over five hours (Blatzinger *et al*, 1981, Chadwick *et al*, 1977, Maxton *et al*, 1986, Sundqvist *et al*, 1980, Ukabam *et al*, 1984). This represents a 100-fold increase in permeation rates compared with lactulose and ^{51}Cr-EDTA (sections

1.4.2.1 and 1.4.2.4). When administered intravenously, however, only between 26% and 69% (depending on the molecular weight of the individual polymer) is recovered in the urine, compared with close to 100% recovery for both lactulose and ^{51}Cr-EDTA (Maxton *et al*, 1986). In one study neither cetrimide nor hyperosmolarity affected PEG permeation rates, whereas permeation of lactulose and ^{51}Cr-EDTA was greatly increased following both interventions (Maxton *et al*, 1986).

In addition, PEG appears to behave in a different manner to lactulose and ^{51}Cr-EDTA in the presence of intestinal disease. From Chadwick's original study, and the subsequent work of other investigators, it can be seen that PEG permeation is markedly reduced in the presence of poorly controlled coeliac disease (Chadwick *et al*, 1977, Ukabam *et al*, 1984). This is in direct contrast to the significant increase in lactulose and ^{51}Cr-EDTA permeation seen in this disease (section 1.5.1). A reduction in PEG permeation has also been demonstrated in Crohn's disease (Magnusson *et al*, 1983), another condition associated with increased oligosaccharide and ^{51}Cr-EDTA excretion, although this is not a universal finding (Hollander, 1992).

Two theories have been put forward to explain these major discrepancies between the permeation of PEG and that of other similar-sized markers. The first is to do with PEG's lipid solubility, and the second concerns the shape adopted by PEG polymers.

Unlike sugars or ^{51}Cr-EDTA, polyethylene glycol demonstrates significant partition into hexane and petroleum spirit, indicating that it possesses a degree of lipid solubility

(Menzies, 1984, Ukabam *et al*, 1984). In quoting thesis work by Laker, Menzies states that PEG can enter liposomes, which have no aqueous pores (Menzies, 1984). Additionally, PEG molecules are able to enter erythrocytes (in common with rhamnose, but not lactulose). These findings have led several authors to conclude that the main route of PEG permeation across the intestinal wall is by direct penetration of the enterocyte lipid membrane (as a function of lipid solubility) rather than non-mediated diffusion via aqueous pores (Bjarnason *et al*, 2002, Maxton *et al*, 1986, Menzies, 1984).

The second theory concerning the route of permeation of PEG polymers concerns their molecular shape. Hollander *et al*, in 1988, used a complex computer modelling program to obtain representations of the molecular structure of several of the commonly used permeability probes, including lactulose, L-rhamnose, ^{51}Cr-EDTA and PEG 400 (Hollander *et al*, 1988). The authors also calculated molecular surface area and minimum cross-sectional diameter. Whilst the majority of probes were globular in shape, PEG polymers appeared to take up a helical form, giving a very small "end-on" molecular diameter. The smallest cross-sectional diameter of PEG was less than that of even the monosaccharides, despite PEG 400 having a molecular weight of more than twice that of these probes. When molecular diameter thus obtained was plotted against published permeability rates of the various probes investigated, a strong negative correlation was seen ($R^2 = 0.98$). Thus the high rate of PEG permeation in healthy volunteers, its permeation into erythrocytes, and the reduction in permeation seen in intestinal disease could all be accounted for if PEG behaved in a similar manner to the small

monosaccharide probes, permeating freely through small aqueous pores, as a function of its helical shape (Hollander, 1992).

There is still debate as to the route of PEG permeation and the usefulness of PEG as a marker of intestinal permeability. Many authors have questioned the applicability of PEG to studies of gut barrier function (Bjarnason *et al*, 2002, Maxton *et al*, 1986, Menzies, 1984). Ukabam and Cooper, after investigationg PEG permeation in patients with coeliac disease concluded that "PEG 400 is not a suitable passive permeability marker to study small intestinal disease because of its lipid solubility" (Ukabam *et al*, 1985) In view of this, PEG has not been used as a marker of intestinal permeability in the current study, and discussions of intestinal permeability focus mainly on the use of oligosaccharides and ^{51}Cr-EDTA, which almost certainly permeate via aqueous pores in a paracellular manner (section 1.4.1.2).

1.4.2.4. Chromium Labelled Ethylenediaminetetraacetic Acid (^{51}Cr-EDTA)

^{51}Cr-EDTA (MW 340) is a gamma-emitting radioisotope that was initially used to quantify glomerular filtration rate (Jenkins *et al*, 1991). It has been used extensively in studies of intestinal permeability. Table 2 shows 24-hour urinary excretion rates in healthy controls and patient groups in a selection of clinical studies.

Following intravenous administration, 24-hour urinary excretion approaches 100% (Bjarnason *et al*, 1994, Maxton *et al*, 1986). ^{51}Cr-EDTA is not hydrolysed within the intestinal lumen or degraded by colonic bacteria, and so 24-hour urinary excretion represents whole gut permeability. Five-hour excretion rates have been measured by some investigators in an attempt to quantify purely small bowel permeability, but colonic absorption probably contributes to five-hour excretion (Travis *et al*, 1992). It is not valid to use ^{51}Cr-EDTA in combination with a monosaccharide as part of a dual-probe technique, as the monosaccharide (but not ^{51}Cr-EDTA) will be affected by bacterial degradation, resulting in an unpredictable excretion ratio (Travis *et al*, 1992). Administering ^{51}Cr-EDTA in hyperosmolar solution results in a mild increase in urinary excretion (Bjarnason *et al*, 1994).

The effective dose equivalent of radiation received following a typical oral dose of 100uCi is <0.12 milliSieverts, which is a clinically negligible amount (Bjarnason *et al*, 1995). Nonetheless, the fact that ^{51}Cr-EDTA is a radioactive source is an important factor limiting its usefulness in everyday clinical practice. The half life of ^{51}Cr-EDTA is 27 days, which is inconvenient if tests are being performed infrequently and means that

urine cannot be stored for more than 2-3 weeks prior to analysis. Analysis is quick and easy to perform; aliquots of urine are simply assayed in a gamma counter along with a standard (usually a dilution of the oral test solution).

Table 2. Twenty-four hour ^{51}Cr EDTA excretion in patients and healthy volunteers

Authors	Dose	N Healthy Controls	Excretion (%)	Hyper-osmolar?	N Study Group	Diagnosis Study Group	Excretion (%)
Bjarnason 1994	–	10	1.1 (mean) SD 0.31	No	9	Coeliac – untreated	2.26 SD 1.53
					16	Coeliac – treated	1.44 SD 0.54
Elia 1987	30μCi	22	1.2 (mean)	Yes	3	Ileostomists	0.41 (mean)
Jenkins 1988	25μCi	50	1.17 (med)	Yes	15	Small Bowel Crohns	3.47 (med)
					19	Colitis	6.07 (med)
Aabakken 1989	3.7MBq	65	2.45 (med) CI95 (2.11-2.86)	No	18	Intra-duodenal	2.38 (med)
Jenkins 1992	50μCi	–	–	Yes	10	Hyperosmolar	Approx 5.5
				No	11	Colitis	Approx 5
Pironi 1990	100μCi	46	2.25 (mean) SD 0.68	No	13	Crohn's small bowel	5.48 SD 2.49
					13	Crohn's ileocolic	4.84 SD 2.83
					6	Crohn's colonic	4.76 SD 2.19
					20	Crohn's resection (relapsing)	4.40 SD 1.8
					11	Crohn's resection (remitting)	3.91 SD 1.7

(Continued)

Authors	Dose	N Healthy Controls	Excretion (%)	Hyper-osmolar?	N Study Group	Diagnosis Study Group	Excretion (%)
Ainsworth 1989	100µCi	28	1.95 (med)	No	15	Crohn's	2.94 (med)
					20	First-degree relatives	1.935 (med)
Jenkins 1991	50µCi	12	2.27 (mean) SEM 0.15	No	6	Ileostomists	0.45 SEM 0.08
					9	NSAID use	4.64 SEM 1.2
Maxton 1986	100µCi	10	1.16 (mean) SEM 0.084	No	10	Cetrimide	6.195 SEM 0.478
				Yes	10	Hyperosmolar stress	2.723 SEM 0.22
Bjarnason 1983	100µCi	22	1.88 (mean)	No	10	Coeliac disease (remission)	4.43 (mean)
					6	Coeliac disease (relapse)	8.16 (mean)

Med: Median
SD: Standard Deviation
CI95: 95% Confidence Interval
SEM: Standard Error of Mean

1.5. Intestinal Permeability in Disease

1.5.1. Coeliac Disease

Patients with coeliac disease were not investigated in the current study. However, coeliac provided the first pathological model for investigating intestinal permeability, and the results of early investigations have played an integral part in the subsequent understanding and evolution of intestinal permeability testing. For this reason the topic is briefly discussed below.

Coeliac disease is an inflammatory disorder of the small intestine characterized histologically by loss of villous height and crypt hypertrophy (Jewell, 2003). It is induced by the prolamins of wheat, barley and rye, and exclusion of these substances in the diet leads to resolution of both symptoms and histological features. This has led to the alternative name "gluten-sensitive enteropathy." Clinically, coeliac disease presents as malabsorption (classically failure to thrive in infants), anaemia, abdominal bloating or discomfort and altered bowel habit (Jewell, 2003). Diagnosis is by small intestinal biopsy, with or without serological tests for antibodies against various cereal antigens.

Investigations in the 1960s revealed that patients with coeliac excreted high levels of urinary disaccharides, amino acids and peptides (Gryboski *et al*, 1963, Kowlessar *et al*, 1964, Weser *et al*, 1965). This was initially thought to represent a primary problem of hyper-excretion, but Menzies and others demonstrated in the late 1970's that the oral

administration of disaccharides such as lactulose and cellobiose resulted in a marked increase in urinary excretion compared with control subjects. This implied that the hyperdisaccharidiuria previously documented was related to increased intestinal permeability, which resulted in increased absorption of sugars from the gastrointestinal tract and consequently increased renal excretion (Cobden *et al*, 1978, Menzies, 1972, Menzies *et al*, 1979, Wheeler *et al*, 1978).

In contrast, the absorption of small probe molecules such as mannitol and L-rhamnose was reduced in patients with coeliac, compared with controls (Cobden *et al*, 1978, Pearson *et al*, 1982). This provided the basis for measuring disaccharide/monosaccharide excretion ratios (the "dual sugar" test), which increased test sensitivity. The use of hyperosmolar test solutions was also found to increase sensitivity (Wheeler *et al*, 1978).

These findings were instrumental in the development of the "para- *versus* trans-cellular" theory of probe permeation, and consequently the use of dual-probe techniques for the measurement of intestinal permeability (section 1.4.2.2). It was postulated that the increase in disaccharide excretion was secondary to increased paracellular permeability, induced by mucosal inflammation. The reduction in monosaccharide excretion was thought to be secondary to reduced mucosal surface area related to villous atrophy (Menzies, 1984).

Over the last 20 years there have been a number of further studies investigating intestinal permeability in patients with coeliac disease. These have confirmed that excretion of

larger probes (lactulose and ^{51}Cr-EDTA) is increased in untreated coeliac disease, and that this is partially resolved following an exclusion diet. Excretion of smaller monosaccharide probes, and of PEG 400, is reduced in coeliac (Behrens *et al*, 1987, Bjarnason *et al*, 1994, Bjarnason *et al*, 1983, Uil *et al*, 2000, Ukabam *et al*, 1984, van Elburg *et al*, 1993). The lactulose/mannitol permeability test has been proposed as an ideal screening test for coeliac, however, it is not commonly used due to the availability of serological tests (Juby *et al*, 1989).

1.5.2. Inflammatory Bowel Disease

Small intestinal, whole gut, and colonic permeability have all been investigated in patients with inflammatory bowel disease and their first degree relatives.

1.5.2.1. Small Intestinal Permeability

There is little doubt that permeability to lactulose is increased in Crohn's disease (Hollander, 1992). In a study of 28 patients with Crohn's of mixed distribution, mean five-hour excretion of lactulose was 0.93% compared with 0.37% in controls ($p<0.05$) (Teahon *et al*, 1992). L-rhamnose excretion was essentially unchanged. In a study by Meddings' group in 1993 these findings were replicated using lactulose and mannitol as permeability probes (May *et al*, 1993). Thirty six Crohn's patients and 31 healthy volunteers were assessed, along with 38 first-degree relatives of Crohn's patients. Mean five-hour lactulose excretion in the Crohn's group was 0.26%, compared with 0.15% in controls ($p<0.01$). Mannitol excretion was similar in the two groups. In a large study from Vienna, 72 patients with quiescent, untreated Crohn's underwent lactulose/mannitol testing, along with 30 healthy volunteers (Wyatt *et al*, 1993). Mean five hour lactulose excretion was 0.5% in Crohn's patients, *versus* 0.22% in controls ($p<0.01$). Mannitol excretion rates were 12.7 and 11.5%, respectively. These patients were then followed for one year, and clinical relapse recorded. Patients were classed into two groups – those with a normal L/M ratio (taken to be < 0.03), and those with L/M ratio greater than 0.03. Of 37 patients with an abnormal initial L/M ratio, 26 (70%) relapsed within one year. Of 35 patients with a normal permeability index, only six (17%) relapsed ($p<0.001$). Using

these data the authors calculated that a single abnormal lactulose/mannitol permeability test had sensitivity for prediction of relapse of 81%.

In contrast to the above studies, Menzies' group, in 1992, showed that only two out of 11 patients with "pure" colitis (four Crohn's, seven Ulcerative colitis) had abnormal permeability to lactulose over 24 hours (Jenkins *et al*, 1992). In these patients small bowel pathology had been excluded by radiological or surgical means.

1.5.2.2. "Whole Gut" Permeability

^{51}Cr-EDTA and PEG probes, when administered orally, are absorbed throughout the length of the gastrointestinal tract (Bjarnason *et al*, 1995, Meddings, 1997, Travis *et al*, 1992). It can be seen from Table 2 that "whole gut" permeability to ^{51}Cr-EDTA is significantly raised in patients with Crohn's disease of unspecified site (Ainsworth *et al*, 1989), as well as in patients with disease limited to the small bowel (Jenkins *et al*, 1988, Pironi *et al*, 1990), the ileo-colonic region (Pironi *et al*, 1990), and the colon (Jenkins *et al*, 1992, Jenkins *et al*, 1988, Pironi *et al*, 1990). It appears from Pironi's study that raised permeability persists throughout periods of clinical remission.

1.5.2.3. Colonic Permeability

At present there is no probe which, when administered orally, gives a measurement purely of colonic permeability. In order to overcome this problem, several authors have administered permeability test probes directly into the large bowel, and measured urinary excretion. In a study by Jenkins *et al* in 1988 (Table 2), ^{51}Cr-EDTA was administered to volunteers and patients with active Crohn's both orally and rectally (Jenkins *et al*, 1988). Results of oral administration are discussed above. Twelve healthy volunteers, 15 patients with active Crohn's of the small bowel, eight patients with active Crohn's colitis, and 11 patients with active ulcerative colitis received rectal ^{51}Cr-EDTA. There was no significant difference between patients with small bowel disease and controls (median 24-hour excretion rates 0.93% and 0.74%, p = NS). In contrast, patients with colitis excreted a median of 5.73% of the administered dose. There was no difference between excretion of the probe by patients with Crohn's colitis and patients with ulcerative colitis.

PEG has been used to assess colonic permeability in a similar manner. Olaison *et al*, in 1989, deposited a bolus of PEG 600 in the descending colon at colonoscopy in 10 patients with Crohn's colitis, 15 patients with ileal involvement and 14 control patients (Olaison *et al*, 1989). Average 6-hour urinary excretion of all molecular weights (which ranged from 590 to 942 daltons) was 11.5% in the active colitics, 2.9% in the colitics in remission, 1.0% in patients with ileitis and 0.8% in controls. PEG excretion was significantly higher in the colitics, compared with the other two groups. Perhaps due to small numbers, the difference between colitics in remission and those with active disease was not significant at the 5% level.

1.5.2.4. Permeability in First-Degree Relatives

In one of the first studies of intestinal permeability and Crohn's disease, Hollander *et al* administered polyethylene glycol with molecular weight ranging from 286 to 506 Daltons (PEG-400) to 17 healthy volunteers, 11 patients with quiescent Crohn's disease and 32 of their first and second degree relatives (Hollander *et al*, 1986). Both patients and their relatives demonstrated increased excretion (9.2% and 10.1%, respectively) compared with healthy controls (3.8%). These findings, along with the fact that a positive family history is a significant risk factor for Crohn's, resulted in much speculation about the role of intestinal barrier dysfunction in causing Crohn's. It has been postulated that a genetically-determined increase in permeability allows antigen to reach the submucosa and stimulate inflammation, thus further compromising the epithelial barrier and creating a cycle of chronic, relapsing inflammation (Meddings, 1997). *In vitro* rodent studies have demonstrated that direct injection of bacterial cell wall products into the colonic wall (thus bypassing the epithelial barrier) produce a chronic granulomatous colitis which behaves in a similar manner to Crohn's disease (Yamada *et al*, 1993).

Since Hollander's study, several investigators have administered a variety of permeability probes to relatives of patients with Crohn's (Ainsworth *et al*, 1989, Katz *et al*, 1989, May *et al*, 1993, Ruttenberg *et al*, 1992, Teahon *et al*, 1992). In all of these studies permeability was abnormal in patients with Crohn's disease, but did not differ significantly between control subjects and first degree relatives. The most obvious conclusion from these data is that the positive finding in Hollander's initial study was an example of "type 1" statistical error, and that asymptomatic relatives have normal

permeability. However, May pointed out that whilst mean probe excretion levels were similar in relatives and control subjects, there appears to be a subset of first degree relatives with abnormally high permeability (defined as > mean + 2SD of control subjects). The authors postulate that this small subset might have inherited the "Crohn's genes," and recommend further longitudinal studies to determine whether or not it is this subgroup that eventually develop the disease (May *et al*, 1993).

1.5.3. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a common, chronic condition accounting for 20-50% of referrals to gastroenterologists (Akehurst *et al*, 2001). It is characterized by abdominal pain associated with bloating and a change in bowel habit. In the absence of biological markers of disease, the diagnosis of irritable bowel syndrome is based upon symptom criteria. Diagnostic criteria in common usage include the guidelines introduced by the Rome Working Team (Drossman *et al*, 1999). The symptoms of irritable bowel syndrome are very similar to those of coeliac disease, inflammatory bowel disease and colorectal malignancy, and hence irritable bowel syndrome should only be diagnosed following evaluation to exclude "organic" disease (Camilleri, 2001).

The pathological basis for irritable bowel syndrome remains unclear. Many clinicians maintain that irritable bowel syndrome is a purely psychological disorder, and its onset is certainly associated with stressful life-events and hypochondriasis (Drossman, 1999). However, there are many pointers towards a physiological basis for irritable bowel syndrome. These are well reviewed by Camilleri and Mayer (Camilleri, 2001, Mayer *et al*, 2002). Putative patho-physiological mechanisms include infection (in up to a third of cases the onset of irritable bowel syndrome is associated with an episode of infectious gastroenteritis (Spiller *et al*, 2000)), heightened visceral perception, and allergy. The multiplicity of factors identified in the pathogenesis of irritable bowel syndrome have led to the condition being described as a "biopsychosocial" disease (Drossman, 1998).

Little work has been performed investigating intestinal permeability in irritable bowel syndrome. In a study of 17 children with symptoms of irritable bowel syndrome, Barau and Dupont demonstrated an abnormal lactulose/mannitol ratio in nine patients following dietary challenge with a specific food antigen (Barau *et al*, 1990). In all nine cases, baseline fasting L/M excretion was normal. The foods used during the provocation test were selected on the basis of a suggestive clinical history and/or a positive skin test. In seven out of nine cases, permeability returned to normal after a one-month exclusion diet. Spiller *et al* investigated 10 patients with post-dysenteric irritable bowel syndrome (Spiller *et al*, 2000). These patients had irritable bowel symptoms 8-48 months after an episode of proven infectious gastroenteritis, and had undergone extensive investigation to exclude "organic" gastrointestinal disease. These patients demonstrated elevated lactulose/rhamnose excretion ratios when compared with control subjects (mean 0.06 *versus* 0.0088, p=0.005). Other abnormalities included an increase in rectal intraepithelial lymphocytes and enteroendocrine cells.

In contrast, some authors have used tests of intestinal permeability to discriminate between patients with "organic" disorders, and those with irritable bowel syndrome. Tibble *et al* followed 602 patients newly referred to a gastroenterology clinic (Tibble *et al*, 2002). Of these 602 patients, 263 were diagnosed to have an "organic" disorder (129 small intestinal, 134 colonic), and 339 with irritable bowel syndrome. The authors found that a lactulose/rhamnose ratio of >0.05 differentiated small intestinal disease from irritable bowel syndrome with a sensitivity of 63%, and a specificity of 87%. Other

authors have also used permeability tests to successfully discriminate between patients with "organic" disease and irritable bowel syndrome (Berstad *et al*, 2000).

Thus the evidence regarding intestinal permeability in irritable bowel syndrome is both scant and conflicting. The model of mucosal micro-inflammation due to hypersensitivity to bacterial or food antigens provides a theoretical basis for increased permeability in this disorder, and more research is therefore required.

1.5.4. Critical Illness

Intestinal permeability in patients with a range of gastrointestinal disorders has been discussed above. Tests of intestinal permeability may aid diagnosis, prognosis and understanding of the pathophysiology of diseases such as inflammatory bowel disease, irritable bowel syndrome and coeliac. These disorders are all "primary" disorders of the gastrointestinal tract. In the "gut origin of sepsis" hypothesis, however, it is hypothesized that an initial non-GI insult, for example haemorrhagic shock, leads to secondary gut damage, with resultant translocation of bacteria and endotoxin, and activation of the gut-associated lymphoid tissue (section 1.3).

Intestinal permeability has been studied in patients with a variety of critical illnesses who are thought to be at risk of secondary gut injury and bacterial translocation. A representative sample of these studies is shown in Table 3. It can be seen that acute systemic insult such as trauma, burn, pancreatitis or cardiac surgery results in an increase in small intestinal permeability, as measured by lactulose/ monosaccharide excretion ratios. In two studies involving patients with acute pancreatitis and severe burns, the ratio of PEG 3350 to 400 excretion was used (Ammori *et al*, 1999, Ryan *et al*, 1992). PEG 3350 excretion was raised in these patient groups, without significant elevation of PEG 400. PEG 3350 excretion probably represents paracellular permeability of the whole intestinal tract, and once again it can be seen that PEG 400 behaves differently to other probe molecules of similar molecular weight (section 1.4.2.3). In several studies

intestinal permeability demonstrated a positive correlation with disease severity (Ammori *et al*, 1999, Juvonen *et al*, 2000, Ryan *et al*, 1992).

It appears, therefore, that systemic insult does result in secondary gut injury; that this is detected by tests of intestinal permeability; and that the degree of gut damage is related to the severity of systemic disease. These data support the initial part of the "gut origin of sepsis" hypothesis. However, the null hypothesis is that this increase in intestinal permeability is simply an epiphenomenon, with no clinical significance. In order to test this hypothesis, several authors have investigated the relationship between raised intestinal permeability and subsequent septic events or systemic endotoxin exposure.

In the study by Ammori *et al* (Ammori *et al*, 1999), permeability was measured within 72 hours of the onset of acute pancreatitis (AP) in 85 patients. Patients with AP demonstrated elevation of the PEG 3350 to PEG 400 ratio, which correlated with disease severity. Permeability was significantly higher in patients with severe AP who subsequently developed multiple organ failure or died, compared with other patients in the "severe" group. In a study of 15 burned patients and 11 controls, Ziegler *et al* found that lactulose/mannitol excretion ratios were increased threefold in septic patients, but were similar to controls in non-infected patients (Ziegler *et al*, 1988). Peng *et al* measured lactulose/mannitol excretion in 22 severely burned patients, and found that L/M ratios correlated well with serum endotoxin levels (Peng *et al*, 2001). This finding has also been documented in patients undergoing elective cardiac surgery (Oudemans-van

Straaten *et al*, 1996), and in patients following intervention for obstructive jaundice (Parks *et al*, 1996).

In contrast, several studies have failed to demonstrate any correlation between intestinal permeability and the subsequent development of sepsis in critically ill patients (Harris *et al*, 1992, Pape *et al*, 1994, Roumen *et al*, 1993). Kanwar *et al* measured intestinal permeability pre and postoperatively in 68 patients undergoing gastrointestinal resections (Kanwar *et al*, 2000). Surgery resulted in an increase in permeability, but there was no correlation between sepsis and either pre or postoperative intestinal permeability.

The current data on intestinal permeability in critically ill patients are therefore somewhat confusing. Critical illness appears to result in measurable intestinal damage, but the clinical significance of this remains unknown. In addition, there are very little data in the literature regarding changes in colonic permeability in these patients, as the majority of studies have used lactulose as a permeability probe.

Table 3. Intestinal permeability in the critically ill

Author	Permeability Probes	Number of Subjects	Diagnoses	Main Findings
McNaught 2002	lactulose L-rhamnose	59	Acute pancreatitis	No relationship between L/R ratio and disease severity
Juvonen 2000	L-Rhamnose Xylose	20	Controls	L/R ratio raised in mild *versus* controls and further in severe *versus* mild pancreatitis ($p<0.05$)
	Methyl-glucose	23	Acute pancreatitis	
Ammori 1999	PEG 400 PEG 3350	25 85	Controls Acute pancreatitis	PEG 3350/400 raised in mild *versus* controls and further in severe *versus* mild disease ($p<0.001$).
Parks 2003	Lactulose Mannitol	11 45	Controls Jaundice	L/M ratio significantly raised in jaundice *versus* controls ($p<0.01$)
Johnston 1996	Lactulose Rhamnose	20 20	Controls ICU sepsis	L/R ratio significantly raised in septic patients ($p<0.001$)
Pape 1994	Lactulose Mannitol	6 32	Controls Multiple trauma	L/M ratio significantly increased in patients with multiple trauma ($p<0.001$)
		11 8	Severe trauma Ruptured AAA	

(Continued)

Author	Permeability Probes	Number of Subjects	Patient Diagnoses	Main Findings
Sinclair 1995	Lactulose Rhamnose	20	Post-cardiopulmonary bypass surgery	L/R ratio significantly increased *versus* controls ($p<0.0001$)
Oudemans-van Straaten 1996	Cellobiose Rhamnose	23	Post-cardiopulmonary bypass surgery	Significant postoperative increase in cellobiose excretion, correlated with circulatieng endotoxin ($p<0.01$)
Harris 1992	Lactulose Mannitol	16	ICU admissions	L/M signifincantly raised compared with controls, not related to illness severity
Ryan 1992	PEG 400 PEG 3350	11	>20% burns	PEG 3350 excretion significantly raised compared with controls, proportional to severity of burn
Deitch 1990	Lactulose Mannitol	15	>20% burns	L/M ratio significantly higher than in controls ($p<0.05$)
Zeigler 1988	Lactulose Mannitol	15	Severe burns	L/M ratio significantly raised following burn, associated with sepsis

L/R: Lactulose/rhamnose
L/M: Lactulose/mannitol
PEG: Polyethylene glycol
AAA: Abdominal aortic aneurysm

1.5.5. Patients Undergoing Chemotherapy

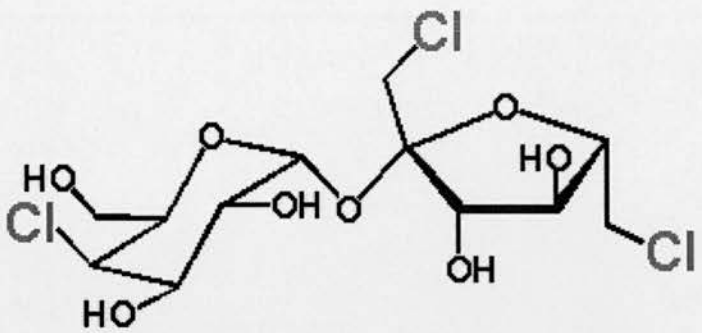
It is well recognised that chemotherapy causes gastrointestinal dysfunction, resulting clinically in nausea, emesis and diarrhoea. Histological changes include mucosal atrophy and "mucositis" (Decker-Baurmann *et al*, 1999, Shou *et al*, 1991, vant Land *et al*, 2002). Studies in humans have demonstrated increased lactulose/ monosaccharide excretion ratios following chemotherapy for soft tissue sarcoma (Fazeny-Dorner *et al*, 2002), haematological malignancies (Parrilli *et al*, 1982, Sundstrom *et al*, 1998) advanced colorectal cancer (Daniele *et al*, 2001), metastatic breast cancer (Parrilli *et al*, 1989) and in patients undergoing high-dose chemotherapy with autologous blood stem-cell transplantation (Johansson *et al*, 1997, Keefe *et al*, 1997). Altered permeability in such patients has been significantly associated with nausea/emesis, stomatitis and diarrhoea (Daniele *et al*, 2001, Fazeny-Dorner *et al*, 2002, Melichar *et al*, 2001). The maximal change in intestinal permeability has been demonstrated to occur between one and two weeks following chemotherapy, permeability returning to baseline levels within a further two weeks (Johansson *et al*, 1997, Keefe *et al*, 1997, Selby *et al*, 1987). Little data is available regarding the effects of chemotherapy on the colon.

1.6. Sucralose and the Triple Sugar Test

1.6.1. Sucralose

Sucralose is a disaccharide of similar molecular weight to lactulose (397.6 Da) (Budavari *et al*, 1989). It was developed as a calorie-free sweetener by McNeil Specialty Products Company, and first introduced into Canada in 1991. Sucralose is now approved for use in over 40 countries around the world. Very small amounts are used to sweeten foods as sucralose is 600 times sweeter than sucrose, from which it is derived. It is marketed under the brand name Splenda™.

Sucralose ($C_{12}H_{19}Cl_3O_8$) is made from sucrose in a five-step process that selectively substitutes three atoms of chlorine for three hydroxyl groups in the sucrose molecule (Grice *et al*, 2000). The molecular structure is shown in Figure 2.



1,6-dichloro-1,6-dideoxy-
beta-D-fructofuranosyl-
4-chloro-4-deoxy-
alpha-D-galactopyranoside

Figure 2. The molecular structure of sucralose

Stability and Pharmacokinetics

Sucralose is a remarkably stable molecule. At high temperatures or under acidic conditions it will slowly hydrolyse to its two constituent chlorinated monosaccharides. At a pH of six and a temperature of 25°C there was no detectable hydrolysis after one year (Grice *et al*, 2000). De-chlorination does not appear to occur. Sucralose is hydrophilic, with a water solubility >25% at 22°C and an octanol/water partition coefficient of 0.3 (Grice *et al*, 2000).

Sucralose is not hydrolysed in the intestinal lumen, and is excreted largely unchanged in faeces (Farhadi *et al*, 2003, John *et al*, 2000, Meddings *et al*, 1998). Initial human studies using an oral dose of ^{14}C-labelled sucralose demonstrated that approximately 85% of radioactivity was excreted in faeces and 11% in urine over five days (total recovery 96%)

(Roberts *et al*, 2000). Over 95% of the radioactive material recovered in urine and faeces was unchanged sucralose, as assessed by thin layer chromatography, HPLC and mass spectrometry. The effective plasma half-life was found to be 13 hours.

Following intravenous administration in dogs and mice approximately 80% of the administered dose was excreted over five days, the vast majority of this occurring within the first 12 hours (John *et al*, 2000, Meddings *et al*, 1998, Wood *et al*, 2000). There have been no published studies investigating urinary excretion following intravenous administration in man.

Toxicity

Extensive toxicity tests have been performed in animals investigating potential side-effects of sucralose. Neither sucralose nor its degradation products have been demonstrated to have a toxic effect upon reproductive capability or the central nervous system, nor any carcinogenic effect, when administered in doses of up to 5000mg/kg (Finn *et al*, 2000, Goldsmith, 2000, Mann *et al*, 2000, Mann *et al*, 2000). In humans no adverse effects were reported following an 8-week course of oral sucralose in doses of up to 500mg/day (Baird *et al*, 2000).

Sucralose as a Permeability Probe

Animal Studies

Sucralose was first used as an intestinal permeability probe by Meddings and Gibbons in 1998 (Meddings *et al*, 1998). In this study damage to selected sites along the GI tract was created using asprin (gastric damage only), indomethacin (gastric and small bowel damage) and trinitrobenzene sulfonic acid (TNBS), administered via a rectal tube (colonic damage only). A test solution containing sucrose, mannitol, lactulose and sucralose was administered orally, and urine collected for 24 hours. It was found that urinary sucrose levels and the lactulose/mannitol excretion ratio were elevated following gastric and small intestinal damage, but not following colonic damage. In contrast, sucralose excretion was elevated following both small intestinal and colonic damage (but not gastric damage).

In a further experiment the intraluminal concentration of the four sugars at various points along the gastrointestinal tract was determined. Following oral administration, all four probes were recovered from the stomach. Lactulose, mannitol and sucralose, but not sucrose were recovered from the small intestine. The only probe molecule to be recovered from the colon was sucralose, which was present in high concentrations up to 18 hours after ingestion.

It was concluded that sucralose was a useful marker of whole gut permeability, detecting both small and large intestinal damage. In the situation of raised sucralose excretion with a normal lactulose/mannitol ratio, colonic damage could be inferred. The authors stated

that "these techniques allow a single screening test that is sensitive to damage at any level of the gastrointestinal tract and may be used in either animals or humans."

Human Studies

There is very little in the literature regarding the use of sucralose as a permeability probe in humans. Meddings' group, in 1997, published in abstract format the results of testing 18 controls and 14 patients with colitis (of unspecified type) with sucralose (2g), sucrose (100g), lactulose (5g) and mannitol (2g). Sucrose and the lactulose/mannitol ratio were the same in both groups, but sucralose excretion in the colitics was almost twice that of controls (3.9% *versus*. 2.2%, $p < 0.01$) (Enns *et al*, 1997).

Suenaert *et al* in 2000, published in abstract format the results of testing 48 volunteers using sucralose (2g), ^{51}Cr-EDTA (50μCi), lactulose (5g) and mannitol (2g). Mean 24-hour sucralose excretion was 2.11 ±1.14%. Twenty-four hour excretion of sucralose correlated with that of ^{51}Cr-EDTA ($r = 0.384$), and 0-6hr excretion of sucralose correlated with the 6hr lactulose/mannitol excretion ratio ($r = 0.452$) (Suenaert *et al*, 2000).

In 2001 Smecuol *et al* published the results of a trial investigating the effect of four different non-steroidal anti-inflammatory drugs (NSAIDs) on intestinal permeability in 19 healthy volunteers (Smecuol *et al*, 2001). Meddings was a co-author. In this trial a combination of sucrose (100g), lactulose (5g), mannitol (2g) and sucralose (2g) was ingested, and urine collection performed for 24 hours. The addition of sucrose made the

test solution markedly hyperosmolar (1800mosmol/L). The median baseline 24-hour excretion of sucralose was 2.01% (CI 95: 1.33-2.78%). Sucralose excretion remained unchanged following two days of therapy with all four NSAIDs, wheras the lactulose/mannitol ratio was increased with three out of four NSAIDs investigated. The authors concluded that the majority of damage caused by modern NSAIDs was to the small intestine.

In April 2003, Suenaert *et al* published the results of a trial investigating the effect of NSAIDs and nicotine patches on gut barrier function (Suenaert *et al*, 2003). Sucralose (2g), lactulose (5g), mannitol (2g) and sucrose (20g) were administered simultaneously in order to assess intestinal permeability. ^{51}Cr-EDTA was administered on a separate occasion. The median 24-hour sucralose excretion in healthy volunteers was 1.81% (IQR 1.09-2.30). Sucralose excretion was increased following the administration of indomethacin, but not the application of nicotine patches. Sucralose excretion during the last 19 hours of collection (6-24 hours post-ingestion) correlated with 6-24 hour ^{51}Cr'EDTA excretion, and was taken to represent colonic permeability.

Analytical Techniques

Due to the chlorination process, sucralose has very different chemical characteristics to other disaccharides. In general, sucralose is harder to detect than other sugar probes. Numerous methods have been used to quantify sucralose in food and body fluids, these include gas chromatography, capillary electrophoresis, radio-labelling using ^{14}C, and HPLC with ultraviolet, mass spectrometry, refractive index and amperometric detection (Farhadi *et al*, 2003, Hatano *et al*, 2002, Kishi *et al*, 2001, Kobayashi *et al*, 2001, Nojiri *et al*, 2002, Zhao *et al*, 2000).

Gas chromatography is a promising new analytical method which may allow the simultaneous quantification of sucralose and other sugar probes (Farhadi *et al*, 2003). This technique has only very recently been described and requires further evaluation. Radiolabelling has obvious limitations in humans. HPLC with refractive index detection was the technique employed in the current study.

1.6.2. The Triple Sugar Test

Sucralose is not fermented in the colon, and is non-toxic (section 1.6.1). These unique properties make sucralose an ideal probe for investigating colonic permeability. Sucralose is a disaccharide with a similar molecular weight to lactulose. These chemical similarities should facilitate direct comparison of urinary lactulose and sucralose excretion rates. As sucralose is stable throughout the gastrointestinal tract, twenty-four hour sucralose excretion should, theoretically, represent "whole gut" permeability.

For the "triple sugar test" used in the current study, a combination of lactulose, L-rhamnose and sucralose was administered orally and urinary excretion of all three probe molecules quantified. The combination of sucralose with lactulose and L-rhamnose should theoretically enable the simultaneous measurement of small intestinal and whole gut permeability. This hypothesis was investigated in the current study.

As "whole gut" permeability is effectively the sum of small intestinal and colonic permeability, it might be possible to obtain a measure of colonic permeability by subtracting small intestinal permeability (measured using lactulose and L-rhamnose) from "whole gut" permeability (measured using sucralose). This hypothesis was also investigated in the current study.

Prior to this study, the triple sugar test of intestinal permeability had not been validated as a measure of "whole gut" or colonic permeability in humans.

Part 2: Methods

2.1. Introduction

The majority of laboratory work in this study was concerned with developing a method for quantifying sucralose in urine. At the time of writing, sucralose was not in use as a permeability probe in the United Kingdom. The author therefore attempted to replicate the method described by Professor J Meddings in Canada (Meddings *et al*, 1998). When this failed, a completely new method (HPLC-RI) was developed, based upon advice from Tate and Lyle UK. All laboratory work concerned with the quantification of sucralose was performed by the author in the Department of Chemistry, University of Hull.

In addition to this original work, a number of other analytical methods were used in this study, namely the quantification of lactulose and L-rhamnose in urine, the quantification of ^{51}Cr-EDTA in urine, and a hydrogen-lactulose breath test of bacterial load in the gastrointestinal tract. These assays were all based upon published techniques, and modified according to local facilities. The quantification of lactulose and L-rhamnose in urine was performed by Dr Simon Fleming, Consultant Biochemist, Treliske Hospital, Truro. All other assays were performed by the author. Clinical studies, including the administration of the triple sugar test to 100 subjects, were performed by the author in Scarborough Hospital, Woodlands Drive, Scarborough, UK.

2.1.1. Ethical Approval

This study was approved by the Scarborough Local Research Ethics Committee. Every participant was given a written information sheet (Appendix 1) and had the opportunity to discuss the study with the author. All subjects were asked to sign a consent form before entering the study (Appendix 2); subjects unable to give consent were excluded from the study. A license for the use of ^{51}Cr-EDTA was obtained from the Administration of Radioactive Substances Advisory Committee (Appendix 3).

2.1.2. Data Storage and Analysis

All data was collected by the author and stored on the author's personal computer as a series of Microsoft® Excel spreadsheets (Microsoft®Corporation, USA). This computer was protected by a password and appropriate "firewall" software (Norton™ Personal Firewall, Symantec™ Corporation, USA). Statistical analyses were performed with XLStatistics (© Rodney Carr 1997-2002) and SPSS® for Windows Version 10.0 (SPSS Inc. Chicago, IL, USA).

2.2. Original Techniques

2.2.1. Sucralose Quantification Using HPLC-PAD

2.2.1.1. Introduction

It was decided to adopt the analytical method described by Professor J Meddings, who pioneered the use of sucralose as an intestinal permeability probe in humans and animals (Enns *et al*, 1997, Meddings *et al*, 1998, Smecuol *et al*, 2001). Medding's method utilised high pressure liquid chromatography (HPLC) with pulsed amperometric detection (HPLC-PAD).

HPLC-PAD is a tried and tested technique for the quantification of urinary sugars such as lactulose, rhamnose and mannitol (Fleming *et al*, 1990, Fleming *et al*, 1993, Miki *et al*, 1996). Amperometric detection offered several potential advantages over other detection methods. Unlike detection using ultraviolet absorption, which requires derivatisation prior to analysis (Nojiri *et al*, 2002), amperometric detection did not necessitate any sample manipulation. HPLC-PAD had been shown to be 10 times more sensitive than HPLC with refractive index detection (Kobayashi *et al*, 2001). HPLC with tandem mass spectrometry was outwith the scope of this study (Hatano *et al*, 2002).

Medding's group reported that sucralose could not be assayed under the conditions commonly used for other carbohydrate permeability probes. This probably reflected the fact that sucralose has a very different chemical structure to other disaccharides, due to

the chlorination process. Medding's group achieved separation using a Dionex Ionpac NS1 column and an acetonitrile/water mobile phase at a flow rate of 1ml/min. This was delivered as a gradient run, the concentration of acetonitrile in water increasing from 0% to 20% over the course of the run. Peaks were identified using pulsed amperometric detection and quantified using peak areas. As the electrochemical detector only functioned at a high pH, post-column addition of 300mmol/L NaOH at a constant flow rate of 0.5ml/min was used. Phenyl-ß-d-thiogalactoside was used as an internal standard (Meddings *et al*, 1998).

In attempting to reproduce Medding's method of quantifying urinary sucralose, equipment and conditions were kept as similar to those described as possible. In particular, a similar Dionex HPLC system with pulsed amperometric detection and an identical column were employed.

2.2.1.2. Reagents

Ultrapure water (Milli-Q system, Millipore, Massachusetts) was used for the mobile phase and aqueous standards. The three mobile phase components were analytical grade water, acetonitrile (Fisher, UK) and 0.1M sodium hydroxide (Fluka, Sigma-Aldrich). These were made up separately, degassed with helium for 30 minutes, and each connected to a computer-controlled pump. Analytical grade sucralose was obtained from McNeil Nutritionals (New Jersey) as a 25% (by weight) aqueous solution. Analytical grade mannitol, L-rhamnose and glucose were obtained from Sigma-Aldrich.

2.2.1.3. Instrumentation

The following HPLC system, manufactured by Dionex (USA) was employed:

- Eluant degas module (model EDM-2)
- Liquid chromatography module (model LCM-3) fitted with Rheodyne automatic sample injector (model 9126)
- Ionpac NS-1 analytical column
- Ionpac guard column
- Advanced gradient pump (model AGP-1)
- Pulsed electrochemical detector with gold working electrode and silver-silver chloride reference electrode
- Advanced computer interface
- Chromatography software (AI-450) running on Dell PC (Intel 486)

Post-column addition of sodium hydroxide was achieved by means of a Gilson HPLC pump (model 302) connected to a Teflon® "T-piece" via a length of pulse-dampening tubing.

A Dionex Carbopac PA-100 anion-exchange column was used in place of the Ionpac-NS1 for the analysis of glucose, galactose, L-rhamnose and mannose.

2.2.1.4. Experimental Technique

Analysis of Mannitol, L-Rhamnose and Glucose in Aqueous Solution

An initial experiment was performed in order to ensure that the HPLC system and integration software were functioning appropriately. The method chosen was one commonly used for the analysis of monosaccharides in the Department of Chemistry, Hull University. An aqueous solution containing mannitol, L-rhamnose and glucose (100mg/L) was prepared. Separation was performed using anion-exchange chromatography on a Carbopac PA-100 column, with an isocratic mobile phase of 0.016M sodium hydroxide at a flow rate of one ml/min. The injection volume was 25µL. The potentials set at the working electrode are shown in Figure 3. No post-column addition was required.

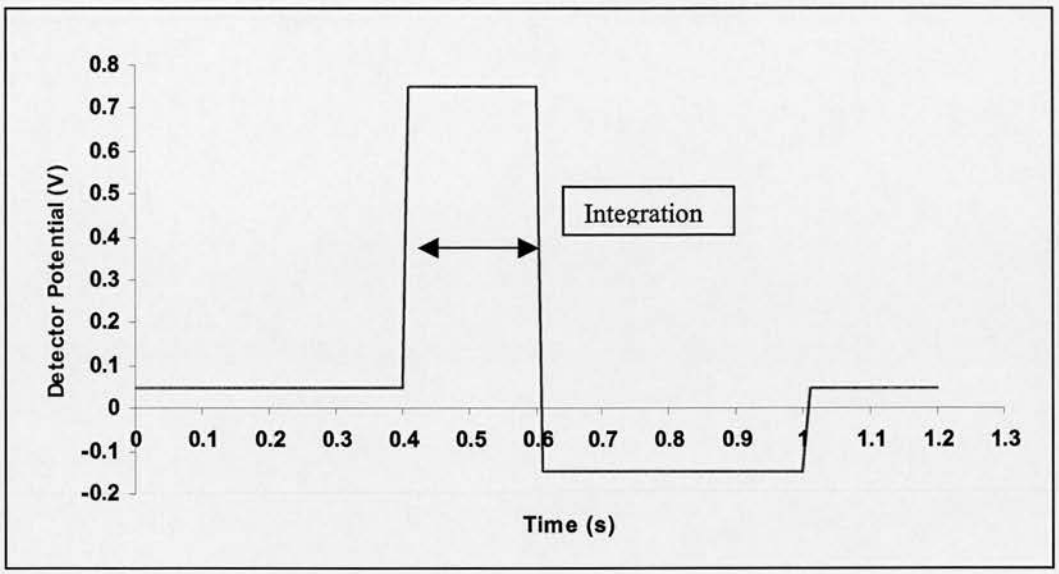


Figure 3. Electrical potentials at the working electrode of the amperometric detector.
A one second cycle was used. The ordinate represents the applied potential at the gold working electrode *versus* the silver-silver chloride reference electrode.

The chromatogram obtained on injection of mannitol, L-rhamnose and glucose is shown below (Fig 4). It can be seen that all three sugars were readily detected at the concentration analysed (100mg/L). Peaks were Gaussian in shape and well-resolved. The component sugars were identified by their retention times, based on current data from the author's laboratory.

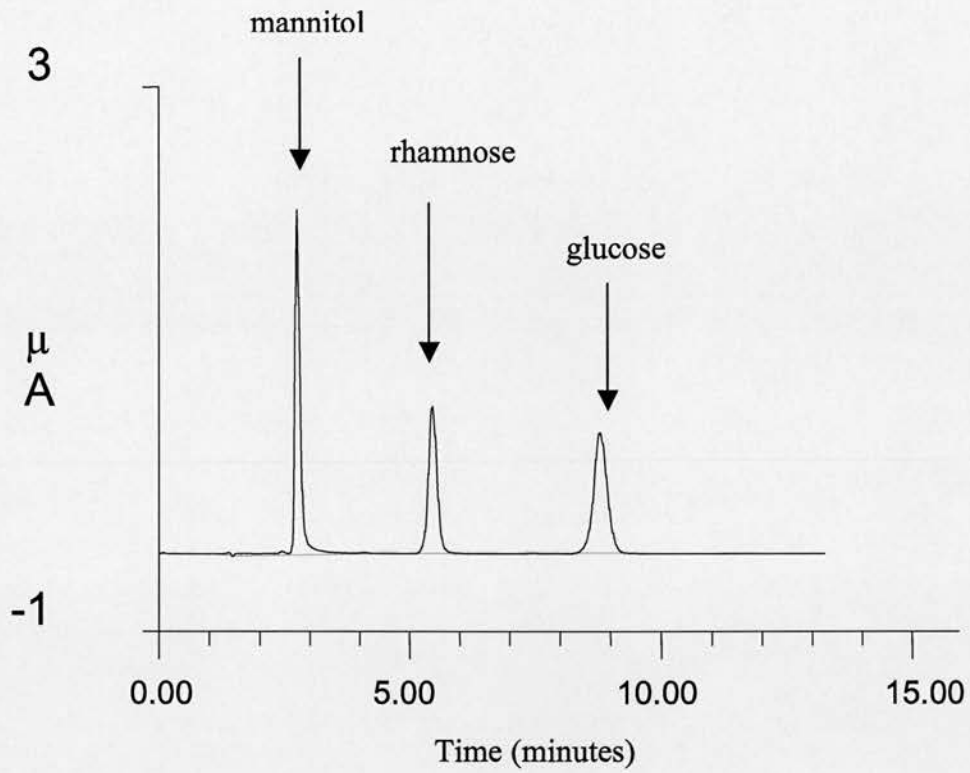


Figure 4. Chromatogram of a monosaccharide solution (100mg/L)

Analysis of Sucralose in Aqueous Solution

Following the successful identification of mannitol, L-rhamnose and glucose, aqueous standards containing sucralose in concentrations 100mg/L and 10g/L were prepared. These were assayed on an Ionpac NS-1 column, using an acetonitrile/water mobile phase. This was delivered as a linear gradient, from 100% water at time 0, to 80% water at 30 minutes. The injection volume was 25μL, and detection was performed in an identical manner to the above experiment. Post-column addition of sodium hydroxide (300mM) at

a flow rate of 1.5ml/min was provided as described by Meddings (Meddings *et al*, 1998). Further runs were performed using an isocratic mobile phase of 20% acetonitrile in water.

No peaks were identified after the injection of sucralose at concentrations of 100mg/L and 10g/L, using either gradient or isocratic mobile phases. The baseline obtained with the post-column addition by HPLC pump was "noisy," however this was within acceptable limits.

Detection of Sucralose, Mannitol, L-Rhamnose and Glucose

There were two possible explanations for this failure to detect sucralose in aqueous solution. Either sucralose was not being eluted from the column, or sucralose was being eluted but not detected by the amperometric system. A further experiment was therefore performed in order to determine whether or not the amperometric detector could register sucralose. The analytical column was removed from the HPLC system, and the distal end of the guard column connected directly to the detector by means of the T-piece, which allowed post-column addition. An isocratic mobile phase of 100% water was used, with post-column addition of sodium hydroxide (300mM) at a flow rate of 1.5ml/min. Litmus paper was used to confirm that the pH of the waste was greater than pH 9. Twenty five microlitres of an aqueous solution containing glucose, galactose, L-rhamnose and mannose in concentrations of 100mg/L was injected and data collected for 30 minutes. The same volume of sucralose (100mg/L followed by 10g/L) was then injected, and data collected 30 minutes.

Injection of a mixture of mannitol, L-rhamnose and glucose in aqueous solution directly onto the detector resulted in a large "spike" deflection at 0.6 seconds, and no further peaks. However, injection of sucralose in concentrations of 100mg/L and 10g/L did not result in any change in the baseline over 30 minutes. It was concluded that under the conditions described by Meddings, sucralose was not detected by the amperometric detector, and the technique of HPLC-PAD was therefore abandoned.

2.2.2. Sucralose Quantification Using HPLC-RI

2.2.2.1. Introduction

Prior to the current study, HPLC with refractive index detection (HPLC-RI) had been used to quantify sucralose, primarily in food products (Kobayashi *et al*, 2001). This method of detection had been shown to be 10 times less sensitive than pulsed amperometric detection. Refractive index detectors, however, tend to be more stable and are not pH dependent. This means that post-column addition of sodium hydroxide is not required when using HPLC-RI.

After failing to detect sucralose with the pulsed electrochemical detector, advice was sought from institutes studying sucralose for nutritional purposes. It was decided to adopt a method described by the Sucralose Research arm of Tate and Lyle (Mary Quinlan, personal communication). This method is summarised below.

Tate & Lyle Method for Quantification of Urinary Sucralose

Urinary samples were prepared by filtration through a 4.5μm syringe filter. No further sample preparation was required. An isocratic mobile phase of 30% methanol in water was used at a flow rate of 1.2ml/min. Separation was performed using a Waters Resolve C18 reversed-phase column (Waters Corporation, Milford, MA, USA), which enabled the use of sample injection volumes of up to 150μL. Peaks were identified using a refractive index detector (Waters model 410), and quantified as peak areas. No internal standard was used. Sucralose elution time was approximately 10 minutes, and limit of detection

was 10ppm. Mean recovery of sucralose in concentrations 12-52ppm was 95-101%, with a coefficient of variation of 2.6-5.3%.

Figure 5 shows an example of a chromatogram obtained by this group after addition of sucralose to urine.

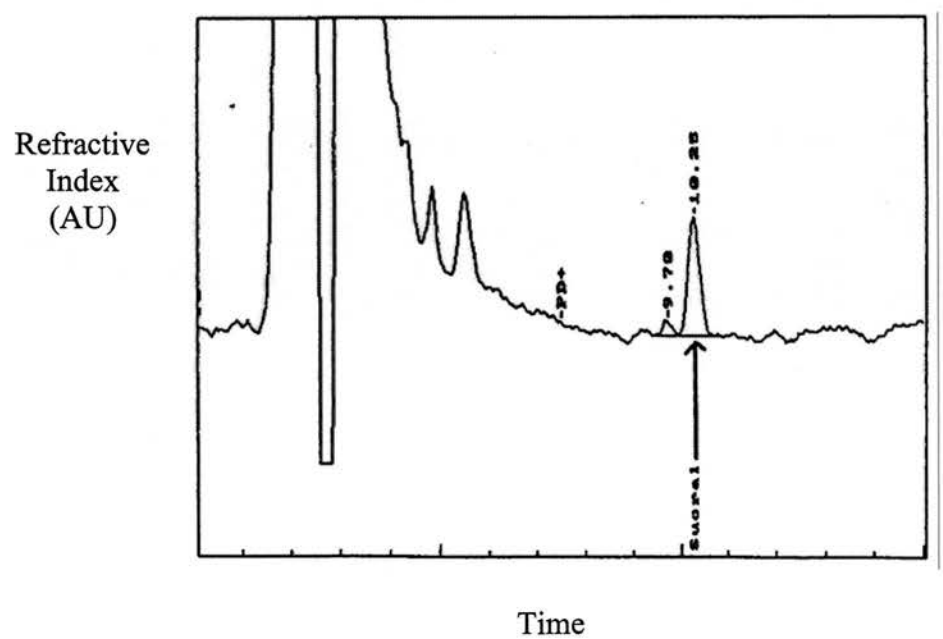


Figure 5. **Tate & Lyle analysis of a urine-based sucralose standard.** The arrow points to the sucralose peak; the numbers represent elution time in minutes (© Tate & Lyle, used with permission).

The following series of experiments represent the author's attempts to replicate this method. It was felt that the use of an internal standard would facilitate greater accuracy in sucralose quantification and would help to control for variability in sample injection

volumes. Phenyl-β-d-glucopyranoside (Figure 6) was suggested as an appropriate internal standard by Dr Juan Navia of the Research and Technical Evaluations wing of McNeil Nutritionals (personal communication).

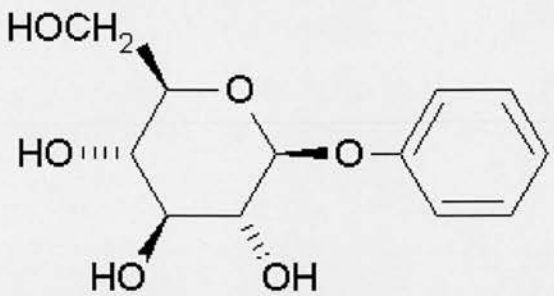


Figure 6. Chemical structure of phenyl-ß-d-glucopyranoside ($C_{12}H_{16}O_6$).

2.2.2.2. Reagents

The mobile phase was made up using HPLC grade methanol (Fisher, UK) and "ultrapure"water (Milli-Q water purification system, Millipore, Massachusetts). One litre was made up on each occasion and de-gassed in an ultrasound bath for at least 30 minutes. Sucralose was obtained from McNeil Nutritionals (New Jersey) as a micronized power. Analytical assays performed by McNeil demonstrated 98-102% purity. Standards were obtained by serial dilution of a 5g/L aqueous standard. The internal standard was analytical grade phenyl-ß-d-glucopyranoside, obtained as a powder from Fluka (Sigma-Aldrich). Aliquots were drawn from a stock solution (10g/L) for addition

to analytical samples. Fresh stock solutions of sucralose and internal standard were made up every two weeks, and stored at 4^0C (section 2.2.2.4).

2.2.2.3. Instrumentation

Chromatography was performed using an HPLC system equipped with an autosampler (PerkinElmer ISS-100, PerkinElmer Inc, Wellesley, MA, USA), a reciprocating piston HPLC pump (Kontron 420, Eching, Munchen, Germany), and a refractive index detector (Gilson 133, Gilson Inc, Middleton, USA). Separation was performed on a reverse phase C18 column (Luna C18(2), 250 x 4.6mm, particle size 5µm, Phenomenex Torrance, CA, USA). A guard column was used (Phenomenex SecurityGuard™ Cartridge). Data from the detector was sent to a data collection unit (PL-DCU, Polymer Laboratories Inc, Amherst, MA, USA) and integration performed with an Intel 386 PC running Polymer Laboratories software PL LC/GC Version 2.0. Data from the detector were also recorded with a chart recorder (BD 40, Kipp and Zonen, Saskatoon, Canada).

Grade A glassware was used in the preparation of aqueous and urine-based standards, and in the processing of patients samples. A Gilson micropipette (P-200, Gilson) was used when volumes smaller than 1ml were required. Calibration was checked by weighing 50 x 100µL aliquots of ultra-pure water. Mean weight dispensed was 0.0993g, with a co-efficient of variation of 2.1%.

2.2.2.4. Experimental Technique

Initial Experiments

Initial experiments, utilising a methanol/water mobile phase and a C18 reversed-phase column, demonstrated that both the internal standard (phenyl-ß-d-glucopyranoside) and sucralose could be detected at the 100mg/L level. The order of elution was phenyl-ß-d-glucopyranoside followed by sucralose.

Selection of Optimal Column Properties

Three different C18 columns were compared. The first of these (column 1) had dimensions of 150 x 4.6mm and a particle size of 5µm (Phenomenex Luna C18 (2)). Column two had dimensions 250 x 10mm and a particle size of 10µm (Spherisorb ODS2). The third column had dimensions 250 x 4.6mm and a particle size of 5µm (Phenomenex Luna C18 (2)). It was found that peak areas were greater for the same concentration of sucralose when using columns two and three compared with column 1. This was assumed to be due to the larger sample injection volumes possible with these columns (up to 100µL). Peaks were better resolved using column three compared with column 2, which might be expected due to the greater efficiency offered by the smaller particle size in column 3. It was therefore decided to use column three (250 x 4.6mm, particle size 5µm). As column three had been used previously in our laboratory a new column of this type and appropriate guard column were purchased and used throughout the remainder of the study.

Optimisation of Detector Sensitivity

Sensitivity of the refractive index detector was increased as far as possible whilst maintaining an acceptably flat baseline. This was judged to have occurred when the sensitivity was set to 1 x 10^{-5} ΔRIU (Refractive Index Units). Increasing sensitivity further resulted in poor resolution of sucralose and internal standard peaks from the baseline. This sensitivity setting was therefore used throughout the remainder of the study.

Optimisation of Mobile Phase

A mobile phase of 30% methanol in water was used, as described by Tate & Lyle (see above). This resulted in good separation of the solvent front, internal standard and sucralose peaks when testing aqueous and urine-based standards. Increasing the concentration of methanol resulted in the internal standard being poorly resolved from peaks caused by unidentified urinary components. Using more dilute solutions of methanol resulted in unacceptable delays in sucralose elution, and so 30% methanol in water was used as the mobile phase throughout the remainder of the study.

Optimisation of Flow Rate

Under the above conditions a flow rate of 1ml/min resulted in the last peak of interest (sucralose) eluting at 12-14 minutes. Pump pressure fluctuated between 160 and 200 bar, and there were no leaks in the system. Faster flow rates resulted in difficulty in resolving the internal standard from peaks caused by unidentified urinary components. In addition, increasing the flow resulted in unacceptably high column pressures. Slower rates resulted in an unacceptably broad sucralose peak, and so a flow rate of 1ml/min was used throughout the remainder of the study.

Chromatograms of Aqueous Standards

Examples of typical chromatograms produced following injection of water (Figure 7) and an aqueous standard containing sucralose and phenyl-β-d-glucopyranoside (Figure 8) are shown below. It can be seen that injection of all aqueous solutions produced a large negative-deflection solvent front beginning at about 2.5 minutes and lasting for 1-2 minutes. Following this the baseline after water injection was flat until the end of the run at 15 minutes. Addition of sucralose resulted in a peak at approximately 12 minutes – the elution time decreased slightly over the course a working day. Addition of phenyl-ß-d-glucopyranoside resulted in a peak at approximately seven minutes. Both compounds produced symmetrical Gaussian-shaped peaks which were well resolved from one-another and readily integrated by the software package.

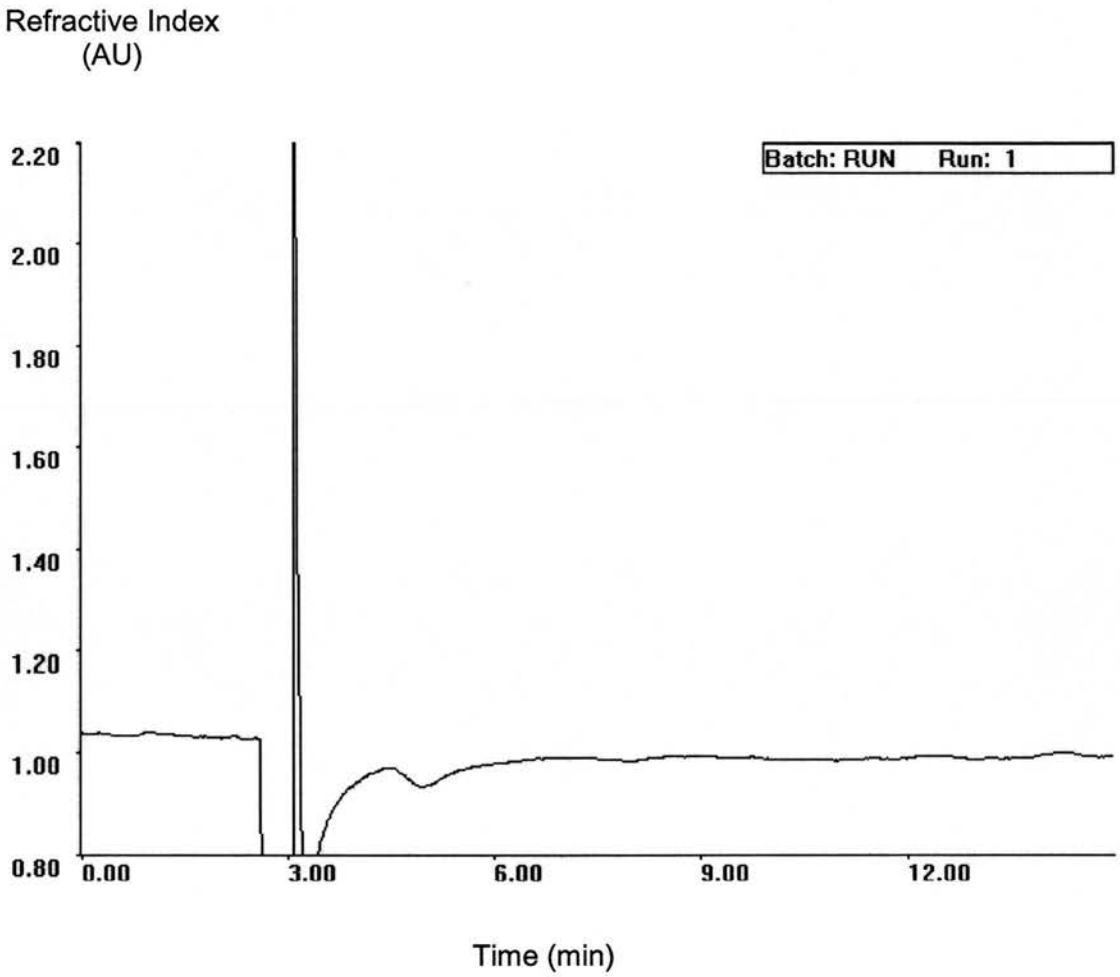


Figure 7. Chromatogram of water "blank"
Note the large negative deflection lasting from approximately 2.5 to 3.5 minutes; this represented the solvent front.

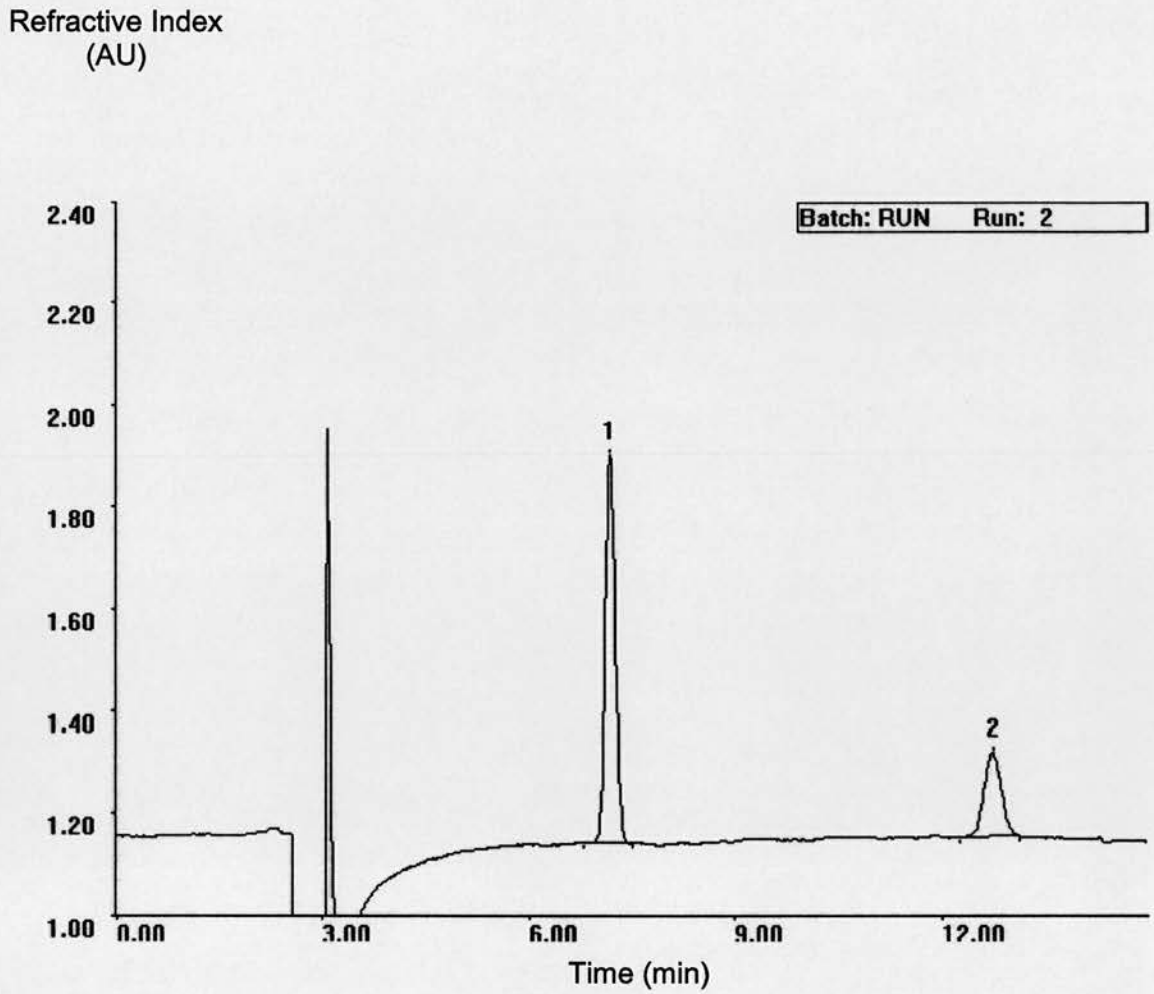


Figure 8. Chromatogram of internal standard and sucralose in aqueous solution.
The two peaks represent phenyl-β-D-glucopyranoside 100mg/L (internal standard, peak 1) and sucralose 50mg/L (peak 2).

Chromatograms of Urine-Based Standards

Examples of typical chromatograms produced following injection of urine blanks and urine-based standards are shown in Figures 9 and 10. Injection of urine resulted in the immediate elution of a number of large, poorly resolved peaks due to unidentified urinary components, the first of which (a negative peak) represented the solvent-front. Whilst each patient's chromatogram was different, in the vast majority of cases an acceptable baseline was regained by six minutes. The addition of sucralose and phenyl-ß-d-glucopyranoside resulted in Gaussian shaped peaks at the same elution times as those seen with aqueous standards. It can be seen that chromatograms obtained after the addition of sucralose to urine were very similar to those demonstrated by the Sucralose Research Arm of Tate & Lyle (Figure 5).

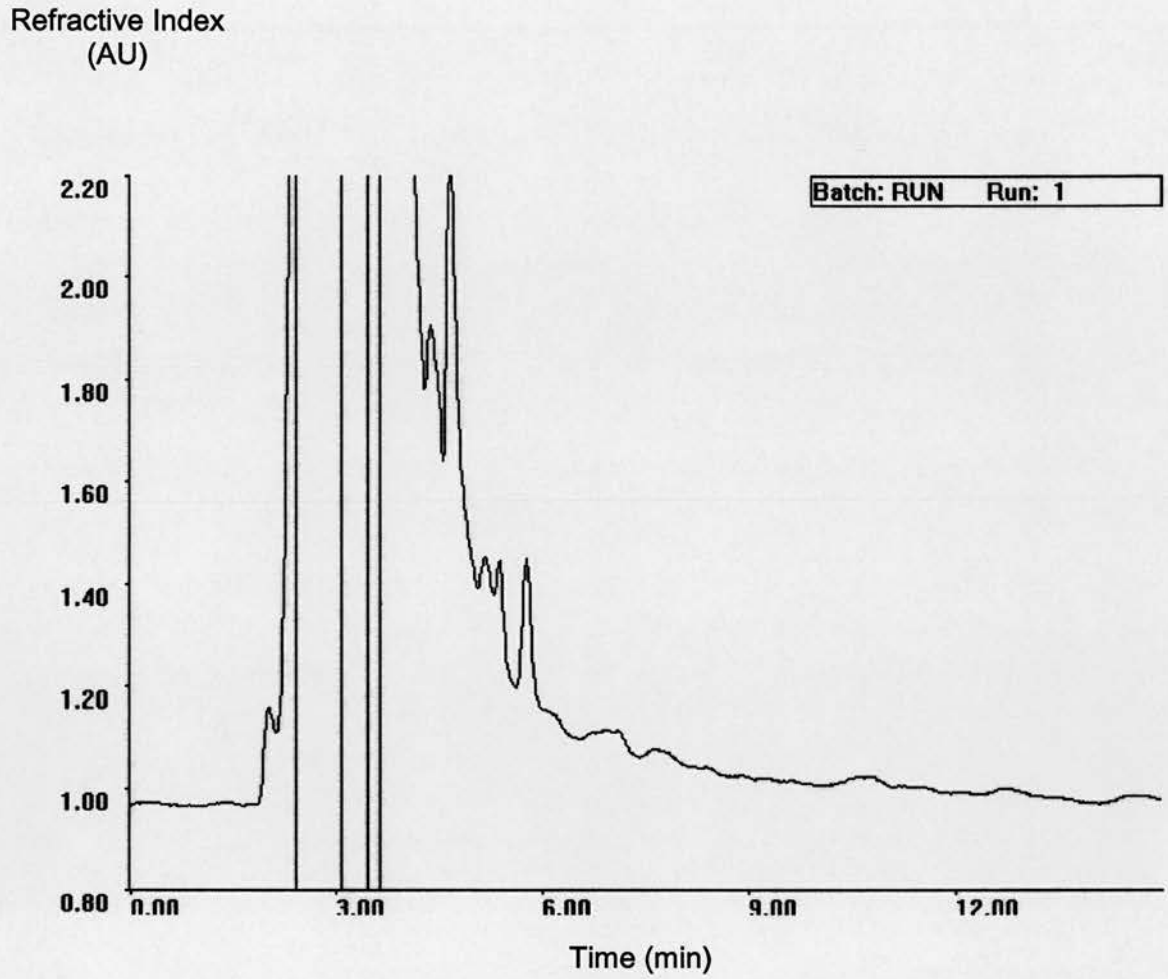


Figure 9. Chromatogram of urine "blank"

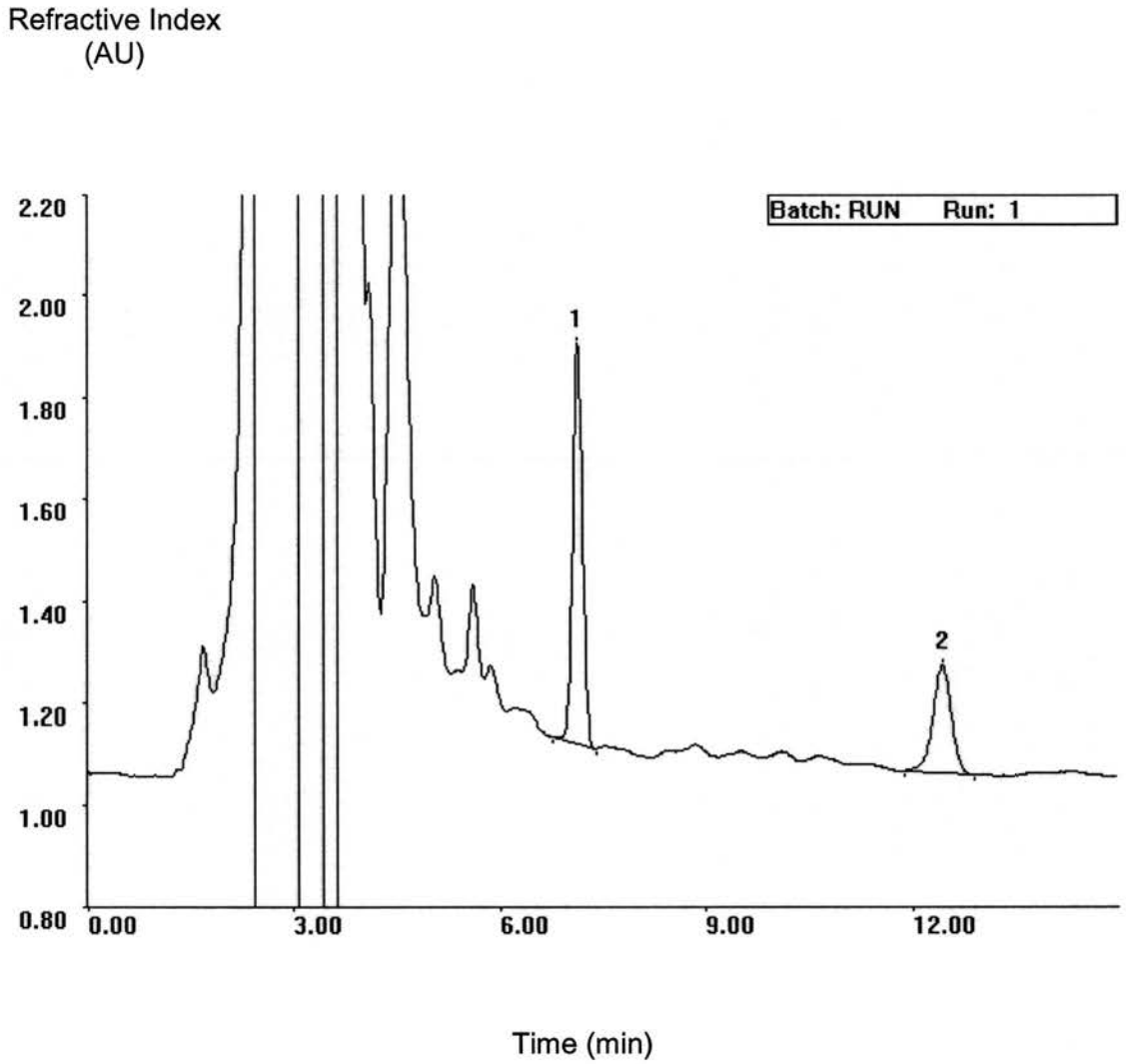


Figure 10. Chromatogram of internal standard and sucralose in urine.
The two numbered peaks represent phenyl-β-d-glucopyranoside 10mg/L (peak 1) and sucralose 50mg/L (peak 2)

Stability Studies

Time-course studies were performed in order to investigate the stability of sucralose in urine and phenyl-β-d-glucopyranoside in aqueous solution. Following ingestion of 5g sucralose, urine was collected for 24 hours in a container with 1ml of 10% thiomersal. This container was kept refrigerated at 4^0C and 10ml aliquots drawn off periodically for analysis. An aqueous solution of 10g/L phenyl-β-d-glucopyranoside (IS) was prepared, and refrigerated in the same manner. 100μL aliquots were drawn off periodically and added to 10ml of water. These were then assayed using the HPLC system described above. Each assay was performed in duplicate, and the mean peak area recorded.

Suralose was stable in urine for the duration of the experiment (165 days). Phenyl-ß-d-glucopyranoside was stable up to 35 days. The sample was next tested on day 56, at which time there was no discernible peak corresponding to the internal standard elution time. A number of unidentified peaks were seen, these were thought to represent breakdown products of phenyl-β-d-glucopyranoside.

It was concluded that sucralose in urine could be safely kept refrigerated for at least a month, and that solutions of internal standard could be safely kept refrigerated for two weeks. These were felt to represent conservative estimates. In light of these data stock solutions of phenyl-ß-d-glucopyranoside and sucralose standards were prepared the week before analysis of patient samples and stored at 4^0C.

Analytical Interference

Aqueous standards containing lactulose, L-rhamnose and thiomersal in concentrations 10 times greater than those normally present in urine following an oral dose of 5g lactulose and 1g rhamnose were assayed. No peaks were identified and there was no interference with sucralose measurement.

Initial Calibration Studies

Calibration was performed using both water and urine-based solutions of sucralose. In each case an initial stock of sucralose 5g/L was made up. Serial dilutions were performed using either water or urine (freshly collected from one individual). To a 10ml aliquot of each standard was added 100µL of internal standard (phenyl-ß-d-glucopyranoside) at a concentration of 10g/L. Samples were then passed through a 0.45µm syringe filter (Alltech Associates, Carnforth, UK) and 100µL of the filtrate injected for analysis. The mean results from two duplicate samples were taken in every case. Linear regression was performed using a statistical analysis package (XLStatistics (© Rodney Carr 1997-2002)).

Calibration curves for sucralose in aqueous solution are shown in Figures 11 & 12. Curves based upon sucralose peak area alone and also the ratio of sucralose to internal standard peak areas are shown. There was a linear association between peak area and sucralose concentration over the concentration range studied (7.8mg/L to 500mg/L). For both sets of data the regression coefficient of the calibration line was greater than 0.995.

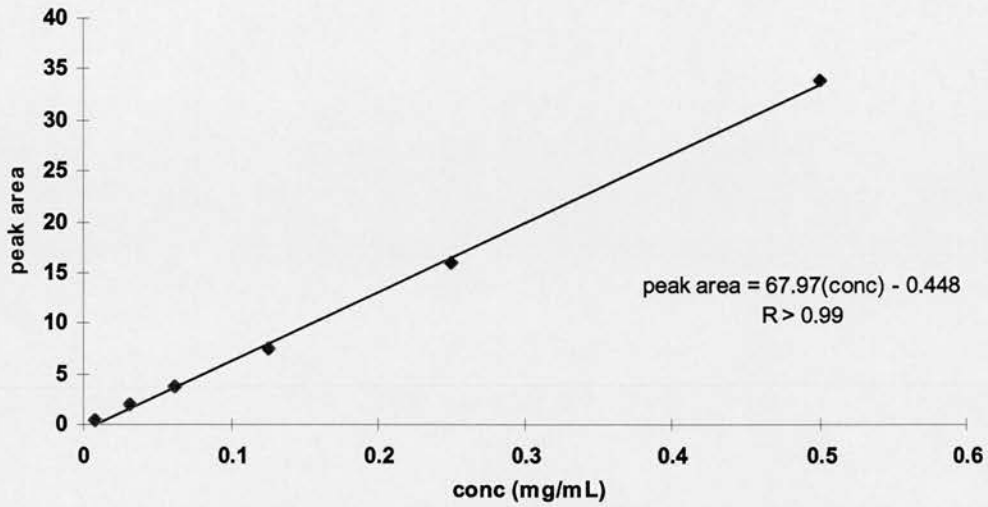


Figure 11. Aqueous calibration based on sucralose peak area alone.
The ordinate represents the peak area for sucralose, measured in arbitrary units.

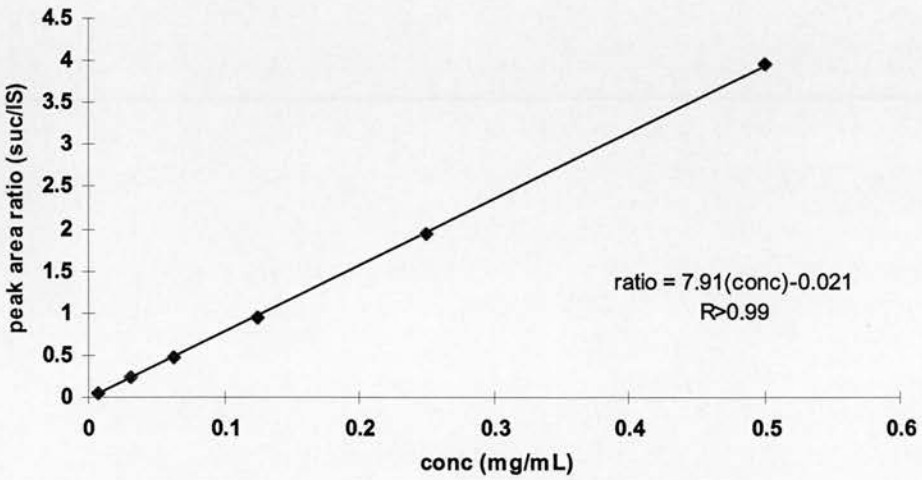


Figure 12. Aqueous calibration based on ratio [sucralose peak area: IS peak area]

Figures 13 and 14 display the same data for urine-based sucralose standards. Sucralose peak area retained a linear relationship to concentration in the range studied (25mg/L to 500mg/L). The regression coefficients of the two calibration lines are both greater than 0.995.

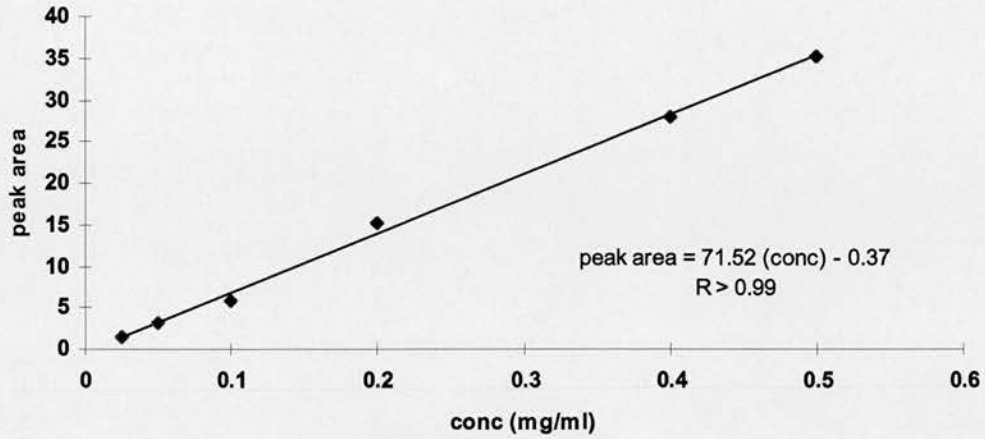


Figure 13. Urine-based calibration based on sucralose peak area alone.
The ordinate represents the peak area for sucralose, measured in arbitrary units.

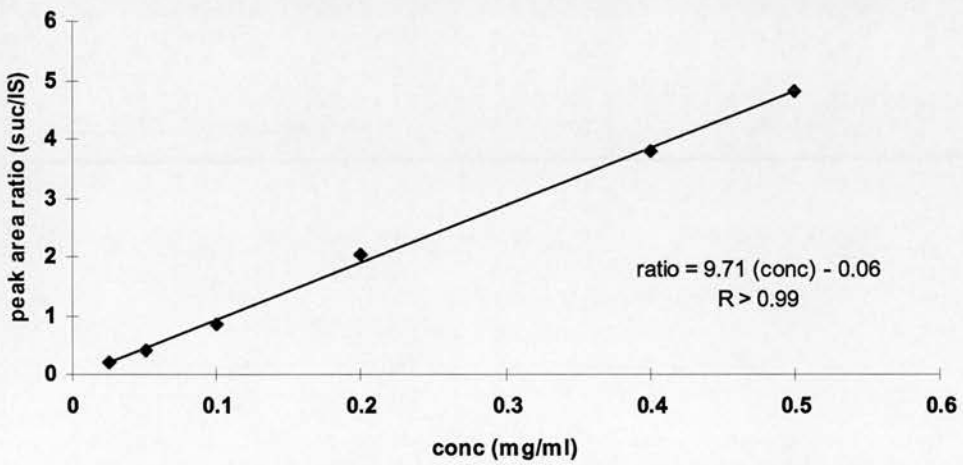


Figure 14. Urine-based calibration based on ratio [sucralose peak area: IS peak area]

Daily Calibration

Following these initial studies, daily calibration using three aqueous standards (in duplicate) was performed prior to the analysis of patient samples or urine-based standards. The equation of the regression line was used to calculate the concentration of sucralose in analytical samples. The statistical package used to calculate the regression line (XLStatistics (© Rodney Carr 1997-2002)) had the facility to omit a constant term – "forcing" the line through the origin. It was found that, when using only three points, it was more reliable to calculate the regression line in this manner than to include a constant term, and so this was adopted throughout the study. Examples of four consecutive calibration curves are shown in Figures 15-18.

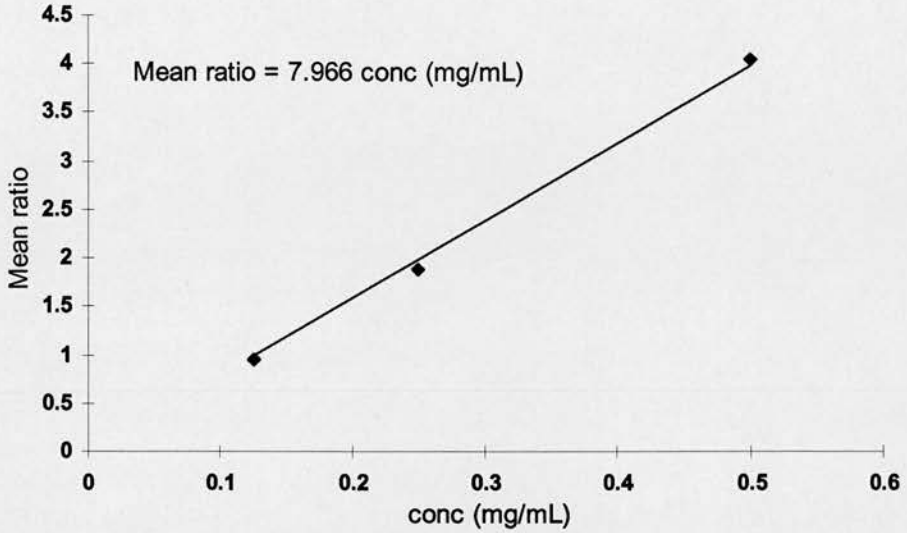


Figure 15. Calibration 1 November 2002.
The ordinate represents the ratio of [sucralose peak area]/[IS peak area].

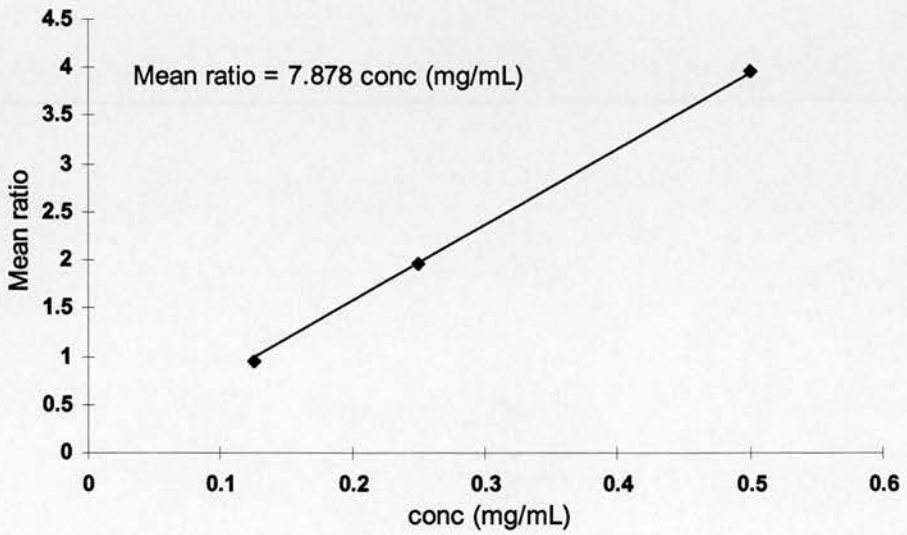


Figure 16. Calibration 8 November 2002.

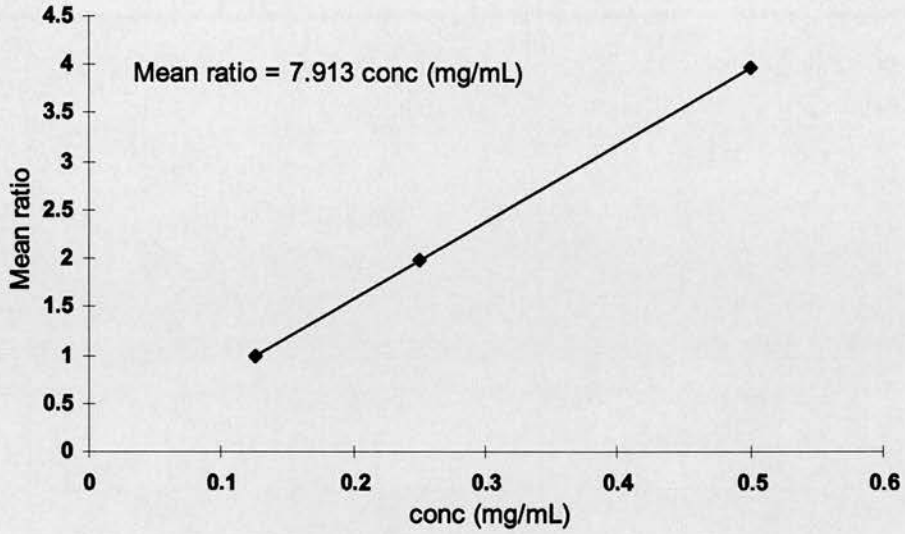


Figure 17. Calibration 15 November 2002

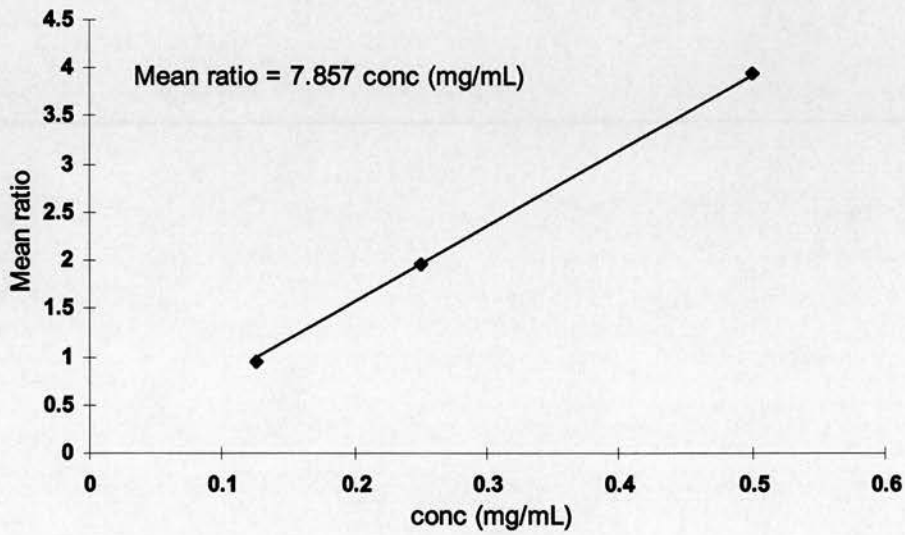


Figure 18. Calibration 22 November 2002.

Limit of Detection

The limit of detection for sucralose in urine was determined using a signal-to-noise method. The minimum detectable sucralose concentration was defined as the concentration at which the peak area was three times that of the area formed by baseline fluctuation (signal-to-noise ratio = 3:1). As the integration software did not have the capacity to automatically integrate the baseline, this was done manually. Twenty chromatograms of urine-based sucralose standards (25mg/L) were analysed. For each run the sucralose peak was integrated, and this area recorded. The peak markers were then moved such that the integrated area included the peak, plus an area of the baseline immediately prior to the peak and of the same width. The difference between the two areas represented the area created by baseline fluctuation. The mean sucralose concentration corresponding to three times this baseline area over 20 runs was 11mg/L (SD = 6.8mg/L). This was taken to be the limit of detection.

Variability of the Method

In order to assess variability of the method, 60 standards of sucralose in urine were made up in the concentration range 25-100mg/L. Each standard was made by adding up to 100µL of aqueous sucralose standard to 10ml of urine. To this was added 100µL of internal standard (phenyl-ß-d-glucopyranoside) at a concentration of 10g/L. Fresh urine was collected from hospitalised patients at random and each standard was made using a different patient's sample.

This work was performed throughout the period of patient samples analysis (approximately one year), in order to provide ongoing quality control. The analytical recovery of sucralose was calculated using the following formula:

% recovery = [(recovered concentration)/(actual concentration)] x 100

Sucralose was well resolved from other urinary components in all of the urine-based standards. Phenyl-ß-d-glucopyranoside (the internal standard) was judged well-resolved from other urinary components in 56 out of 60 (93%) standards. In the remaining four cases the internal standard peak was obscured by unidentified urinary components. The quantification of sucralose in these samples was based upon the peak area of sucralose alone.

The mean recoveries of urinary sucralose standards at the three concentrations studied are shown in Table 4. The mean recovery of the 60 samples was 103.1%, with a coefficient of variation of 6.07%.

Table 4. Analytical recovery of urinary sucralose

Concentration (mg/L)	Number of Samples	Mean Recovery (%)	SD (%)	CV
100	20	105.0	4.47	4.26%
50	20	102.9	5.99	5.82%
25	20	101.5	7.70	7.59%
Overall	60	103.1	6.26	6.07%

SD: Standard deviation
CV: Co-efficient of variation

Analysis of Test Subjects' Urine

Preparation and analysis of test subjects' urine was as described for the urine-based standards. To a 10ml aliquot of each five or 24-hour urine collection was added 100µL of internal standard (phenyl-ß-d-glucopyranoside) at a concentration of 10g/L. Samples were then passed through a 0.45µm syringe filter (Alltech Associates, Carnforth, UK) and 100µL of the filtrate injected for analysis. Duplicate runs were performed in every case. Calculation of sucralose concentration was performed using daily 3-point calibration curves. The ratio of sucralose peak area to internal standard peak area was used whenever possible. In the few cases where the internal standard peak was obscured, the sucralose peak area alone was used. Sucralose excretion was calculated using the following formula:

Percentage Excretion = [(urinary sucralose (g/L)) x (urine volume (ml))] / 50.

2.2.3. Administration of the Triple Sugar Test

2.2.3.1. Composition of the Test Solution

Doses of Test Sugars

The oral solution used for the triple sugar test of intestinal permeability contained 5g lactulose (7.5ml of Duphalac® syrup, Solvay Pharmaceuticals, Inc., Marietta, Georgia, USA), 1g L-rhamnose (analytical grade powder, BDH Laboratory Supplies, Poole, UK) and 5g sucralose (analytical grade micronized powder, McNeil Nutritionals, NJ, USA). The doses of lactulose and L-rhamnose were similar to those used in the majority of studies of intestinal permeability (see Table 1), and had previously been used in the author's institution (McNaught *et al*, 2002).

Authors of previous studies employing sucralose had used a dose of 2g (Enns *et al*, 1997, Smecuol *et al*, 2001, Suenaert *et al*, 2000). Urinary excretion in healthy controls in these studies was approximately 2%, giving a urinary sucralose concentration of 20mg/L, assuming a 24-hour urine volume of 2000ml. It was anticipated that some patients (eg following intestinal resection) might excrete less than 2% of the oral dose, and that the administration of 2g sucralose to these patients might result in urinary concentrations close to the lower limit of detection of the HPLC-RI system (11mg/L, section 2.2.2.4). It was therefore decided to improve analytical accuracy by increasing the dose of sucralose. A dose of 5g was calculated to result in a 24-hour urinary concentration of 50mg/L in healthy controls. Analytical recovery at this concentration was 102.9%, with a coefficient of variation of 5.82% (Table 4). Whilst this dose was higher than that used in

other clinical trials, animal studies had demonstrated no toxicity with repeated doses of up to 5000mg/kg (equivalent to 350g for a 70kg adult).

Osmolality of the Test Solution

The molecular weights of lactulose, L-rhamnose and sucralose are 342.30, 164.16 and 397.64, respectively (Budavari *et al*, 1989). Converting weight to molarity for each sugar, the test solution contained 14.61mmol lactulose, 6.09mmol L-rhamnose and 12.57mmol sucralose (total osmotic content 33.27mmol). The overall volume of the test solution was 150ml, giving a calculated osmolarity of 221.8mmol/L, which is slightly hypotonic to plasma (Haslet *et al*, 1999)).

In addition to calculating approximate osmolarity in this manner, osmolality was measured by analyzing an aliquot of the oral test solution in an osmometer (Advanced™ Micro-Osmometer, Model 3MO Plus, Vitech Scientific Ltd, Partridge Green, West Sussex, UK). Measurement was performed by Scarborough Hospital Clinical Biochemistry department, using a depression of freezing point technique (Kaplan *et al*, 1996). The mean osmolality of three repeat samples was 251 mOsmol/kg (range 250-252 mOsmol/kg).

2.2.3.2. Administration of the Test Solution

Subjects were asked to refrain from alcohol for 24 hours prior to the test and to fast from midnight. The triple sugar tests were administered by the author between 0830 and 0930 hrs the following morning. As concentrated sucralose is unpalatable, subjects were asked to quickly drink 30ml water containing sucralose (5g), immediately followed by 120ml water containing lactulose (5g) and L-rhamnose (1g). Subjects were witnessed drinking the entire test solution. After ingesting the solution subjects were allowed to drink water for the first five hours and to eat and drink freely following this. Subjects were asked to refrain from alcohol until they had completed the 24-hour urine collection.

2.2.3.3. Urine Collection

Urine was collected for 24 hours in two containers, each of which contained 1ml 10% sodium merthiolate as a preservative. Subjects were asked to void urine immediately before the permeability test. Following ingestion of the test solution, urine was collected for five hours in the first container. After five hours subjects were asked to void for the last time into the first container and to use a second container to collect urine for the following 19 hours, voiding for the last time into this container at the end of the 24-hour period.

2.2.3.4. Sample Preparation

Subjects were asked to return the two urine containers to Scarborough Hospital within two days of completing the permeability test. Both containers were weighed on an electronic flat-pan balance (LS 5000, Ohaus, Pine Brook, NJ, USA), the weight of an empty container plus preservative subtracted, and the corrected weight used as a measure of volume, assuming a weight to volume ratio of 1.00. Two 5-10ml aliquots were drawn from the five hour collection and stored at -20^{0}C. The remainder of this collection was then mixed with the 19 hour collection to give a 24-hour sample. Three 10-20ml aliquots were drawn from this and stored at -20^{0}C. The remaining urine was discarded. Prior to analysis urine was thawed at room temperature and transported to the appropriate laboratory (Department of Chemistry, Hull University for sucralose analysis and Department of Clinical Biochemistry, Treliske Hospital, Truro for lactulose and L-rhamnose analysis). On arrival at the appropriate laboratory urine was stored at 4^{0}C until analysis.

2.3. Other Analytical Techniques

2.3.1. Lactulose and L-Rhamnose Quantification Using HPLC-PAD

2.3.1.1. Introduction

The technique used for the quantification of urinary lactulose and L-rhamnose was HPLC-PAD. This technique has been described previously (Fleming *et al*, 1990, Fleming *et al*, 1993). Assays were performed by Dr Simon Fleming, Consultant Biochemist, Treliske Hospital, Truro, UK. Five ml aliquots of five and 24-hour urine collections were stored at -20^{0}C in Scarborough Hospital. Periodically, batches of 30 samples were defrosted at room temperature and a 2ml aliquot drawn from each sample into a screw-topped container. These were posted to Dr Fleming for analysis.

2.3.1.2. Reagants

Deionized water (18MΩ/cm) was produced with an in-house deionizer (Elga, High Wycombe, UK). Zinc acetate, sodium hydroxide and Amberlite resins Ir-120 H^+ and Amberlite IRA 400 Cl^- were obtained from BDH (BDH, Poole, UK), Lactulose and L-rhamnose were obtained from Sigma (Poole, UK), and melibiose (internal standard) from BDH.

2.3.1.3. Instrumentation

The following HPLC system was employed:

- Dionex GP40 gradient pump (Dionex UK, Camberly, UK)
- Gilson 234 autoinjector (Gilson Inc. Middleton, WI, USA)
- Dionex column PAI (4 x 250mm)
- Dionex Carbopac PAI guard column
- ESA model 5040 analytical cell with gold electrode (ESA Inc, Chelmsford, MA, USA)
- PAD detector (Coulachem II no. 2, ESA)
- UniPoint™ LC System Software (Gilson Inc.)

2.3.1.4. Experimental Technique

Mobile Phase Preparation

The mobile phase was a mixture of 0.05mmol/l zinc acetate and 120mmol/l sodium hydroxide. Deionized water (989.4ml) was degassed with helium for 20min. A 1-ml volume of 0.5mmol/L zinc acetate solution was added and mixed. A 9.6ml volume of 50% (w/v) sodium hydroxide was added with degassing for a further 10 minutes.

Analytical Sample Preparation

Depending on collection volume, samples were diluted between 1:10 and 1:40 with deionized water to a volume of 1ml. This dilution was performed on an aliquot of the neat frozen urine. A 1ml volume of internal standard (melibiose 250mg/L) was added and the mixture de-salted with ion-exchange resins (Amberlite IR-120 H^+ and Amberlite IRA 400 Cl^- mixed in a ratio of 1: 2, respectively). Samples were then spun down and the supernatant used for injection.

Chromatographic Conditions

Supernatant (40µl) was injected onto the column and eluted with 120mmol/l sodium hydroxide/0.5mmol/l zinc acetate at a flow-rate of 1ml/min at 20^0C. Detection was with a pulsed electrochemical detector, set to a sensitivity of 20µA. Integration of peak heights was performed with internal standardisation. Percentage excretion of lactulose and L-rhamnose was calculated according to the following formula:

Percentage Excretion =

[(urinary sugar conc (g/L) x urine volume (ml)) / dose administered (mg)]

X 100

A typical chromatogram (obtained from one of the test subjects) is shown in Figure 19.

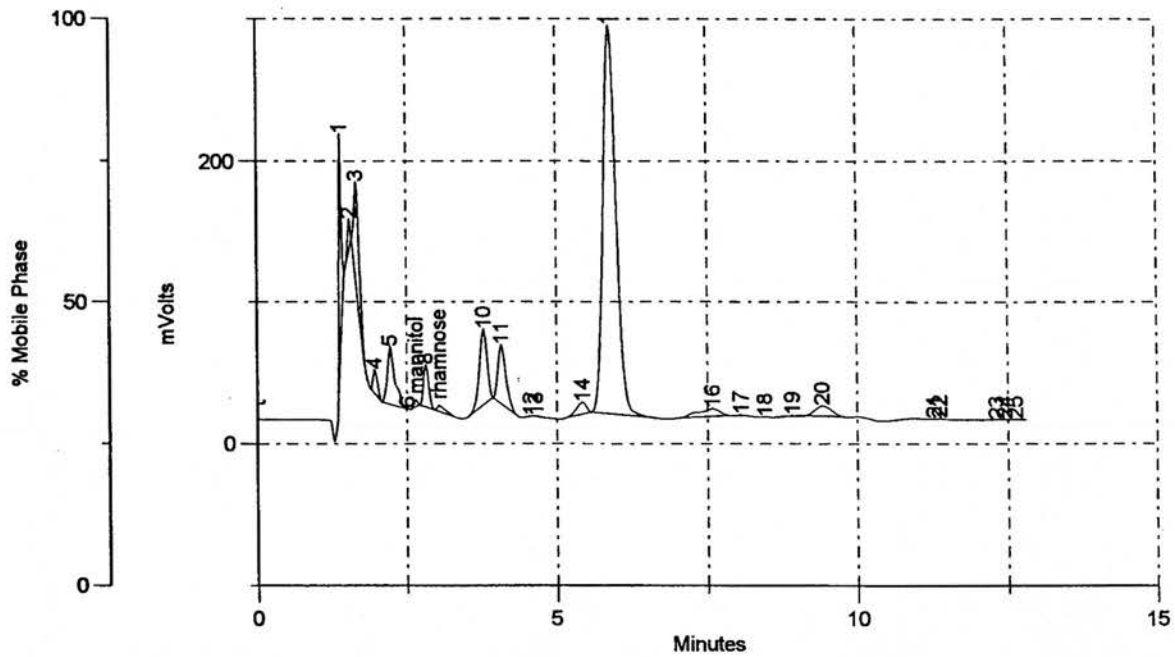


Figure 19. Chromatogram of an analytical sample obtained using HPLC-PAD. Peaks numbers 8, 15 and 20 correspond to L-rhamnose, internal standard and lactuose, respectively.

Analytical Interference

In order to exclude analytical interference between sucralose and lactulose or L-rhamnose, aqueous standards containing sucralose at a concentration over 10 times that expected in subjects urine (1g/L) were injected, and produced no peak or alteration to the baseline. In addition, a series of four lactulose standards was produced in duplicate. To one set of standards was added sucralose (100mg/L). The results of HPLC analysis are shown in Figure 20. It can be seen that the addition of sucralose did not significantly alter calibration with lactulose.

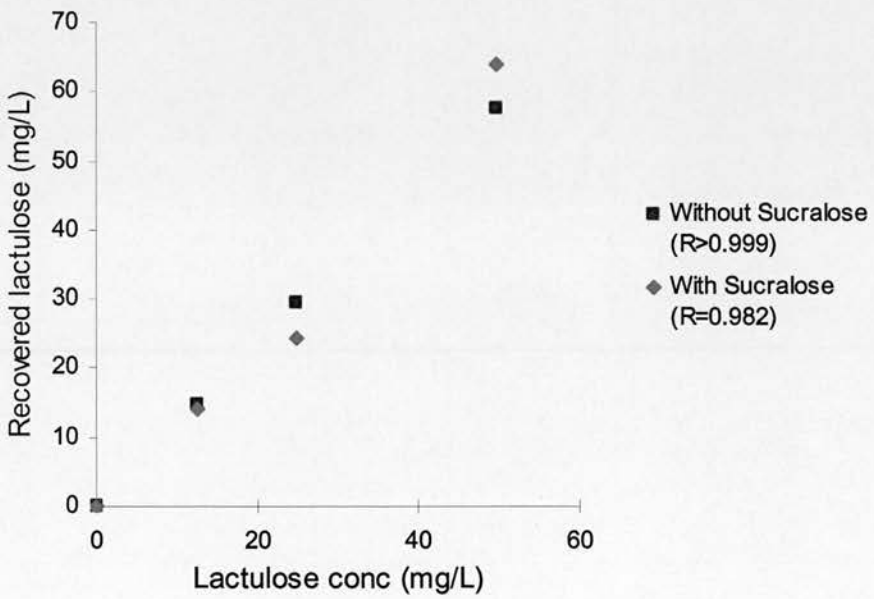


Figure 20. Calibration with two sets of lactulose standards.
To one set of standards was added sucralose (100mg/L).

Daily Calibration and Quality Control

An aqueous standard containing L-rhamnose 50mg/L, lactulose 10mg/L and internal standard was analysed at the beginning of every day's chromatography, and every eight runs following this. The ratio of standard peak height to internal standard peak height was recorded as a means of quality control. Over 17 consecutive runs, the coefficient of variation of such ratios was 5.34% for lactulose and 6.84% for L-rhamnose.

In addition, at least one urine-based standard containing L-rhamnose (50mg/L), lactulose (10mg/L) and internal standard was analysed each day. Over 12 consecutive runs, the mean analytical recovery of L-rhamnose was 108.3% (CV 2.91%) and of lactulose was 100.5% (CV 5.84%).

2.3.2. Lactulose-Hydrogen Breath Test

2.3.2.1. Introduction

Respiratory hydrogen excretion following the ingestion of lactulose was used in this study as a surrogate marker of bacterial colonisation of the GI tract. The measurement of breath hydrogen in tests of GI function has a long history. The basic premise is that bacterial fermentation of sugars results in the production of intraluminal hydrogen, which is absorbed systemically and excreted by the lungs. Initially, gas chromatographic methods were used for the quantification of hydrogen in expired air. These were cumbersome and involved collecting expired air and transporting it to a laboratory (Gilat *et al*, 1978, Rhodes *et al*, 1979). More recently, hand-held hydrogen meters have been developed and validated for clinical use. These allow immediate and accurate quantification of breath hydrogen in the outpatient clinic or at a patient's bedside (Fleming, 1990).

Common Test Sugars

Though basal fasting breath hydrogen levels give some information, response to a sugar challenge is far more informative. The three sugars in common usage are glucose, for detection of small-bowel bacterial overgrowth (Lewis *et al*, 1999, Stotzer *et al*, 2000), lactose, for the detection of lactase deficiency (Brummer *et al*, 1993, Casellas *et al*, 2003), and lactulose, normally used for the determination of oro-caecal transit time (see below). Baseline fasting breath hydrogen levels are recorded, the sugar (usually 5-20g) is administered, and breath hydrogen readings taken at regular intervals for up to four hours. A significant rise in breath hydrogen concentration (often defined as a sustained

rise >20ppm above baseline levels (Fleming, 1990, Salzberg *et al*, 1988) indicates bacterial fermentation of the administered sugar.

A rise in breath hydrogen levels following the ingestion of glucose or lactose is abnormal, as both sugars are usually completely absorbed in the proximal small bowel, before bacterial fermentation can take place. An increase in breath hydrogen levels therefore implies malabsorption in the case of lactose, or small bowel bacterial overgrowth in the case of glucose.

In contrast, a rise in breath hydrogen following the ingestion of lactulose is a normal phenomenon, as lactulose is not hydrolysed in the small intestine, and only a very small amount is absorbed. The majority of ingested lactulose therefore passes unchanged into the caecum, where it is degraded by colonic bacteria, resulting in a rise in breath hydrogen. The length of time from ingestion of lactulose to a rise in breath hydrogen has been shown to correlate with oro-caecal transit time as measured by barium meal and scintigraphy (Caride *et al*, 1984, Hirakawa *et al*, 1988). Previous data regarding oro-caecal transit times in clinical studies are shown in Table 5.

Factors which have been shown to influence oro-caecal transit time include the dose of lactulose ingested (Diggory *et al*, 1985), the method of ingestion (whether ingested in solution or as part of a meal) (Ladas *et al*, 1989) and the "cut-off" breath hydrogen concentration employed (Hirakawa *et al*, 1988). Factors which have been shown not to

influence the test include osmolality of the test solution (Diggory *et al*, 1985), and patient gender or race (Salzberg *et al*, 1988).

A proportion of healthy volunteers do not produce hydrogen after ingesting lactulose, this group also have lower baseline hydrogen levels (Salzberg *et al*, 1988). These individuals have been termed hydrogen "non-producers," and comprise between zero and 21% of volunteers studied (Diggory *et al*, 1985, Gilat *et al*, 1978, Hirakawa *et al*, 1988, Jorge *et al*, 1994, Ladas *et al*, 1989, Salzberg *et al*, 1988).

Table 5. Lactulose-hydrogen breath tests in healthy volunteers

Author	Year	Number of Control Subjects	Mean Oro-caecal Transit time (min)	Number of "Non-Producers"
Gilat	1978	15	N/A	2
Tursi	2003	20	88.2	N/A
Caride	1984	19	75.1	N/A
Saltzberg	1988	34	90 (approx)	8
Diggory	1985	13	82.2	N/A
Jorge	1994	42	82 to 117	3
Ladas	1989	7	81.75	N/A

N/A: Data not available

2.3.2.2. Apparatus

The concentration of end-tidal breath hydrogen in control subjects and ileostomists was measured using a previously validated hand-held detector (Bedfont EC 60 Hydrogen Monitor, Bedfont Technical Instrument, Sittingbourne, UK) (Fleming, 1990). This incorporated a simple end-tidal sampling system (T-piece with two flutter valves), and an electrochemical sensor. Data from the sensor was displayed on a liquid crystal screen on the front of the unit.

Published data regarding the sampling imprecision of the hydrogen meter described an inter-batch coefficient of variation (CV) of 8.0% at a concentration of 25 parts per million (ppm) (Fleming, 1990). Calibration in the current study was performed monthly, using a standard hydrogen mixture in air (200ppm). As in Fleming's study, the unit was found to be exceptionally stable over time at room temperature.

2.3.2.3. Test Protocol

Subjects undergoing the lactulose-hydrogen breath test were fasted from midnight, and were seen by the author between 0830 and 0930 hrs the following morning. A baseline breath hydrogen sample was obtained, and subjects were then asked to drink a solution containing 13.3g of lactulose (20ml Duphalac® syrup, Solvay Pharmaceuticals, Inc., Marietta, Georgia, USA) in 120ml water (calculated osmolarity 324mOsmol/L). Breath hydrogen concentration was measured every 15 minutes for three hours, during which time patients were kept "nil by mouth."

Breath sampling was performed in a standardised manner, as described previously (Fleming, 1990). Patients were instructed to inspire and hold their breath for 15 seconds. They then exhaled completely through the sampling device of the instrument so that the end-tidal breath remained trapped between two flutter valves, allowing it to diffuse into the sensor. The readout from the machine tended to fluctuate over the ensuing minute or so; the highest level reached was recorded.

2.3.2.4. Data Analysis

Data was recorded onto a Microsoft Excel® database, and analysed using XLStatistics (XLent Works, ©Rodney Carr, Allansford, Australia). A rise in breath hydrogen concentration of >20ppm above baseline fasting levels was regarded as significant (Fleming, 1990). The proportion of subjects in control and ileostomy groups exhibiting a significant rise in breath hydrogen was compared using Fishers Exact test.

2.3.3. ^{51}Cr-EDTA Quantification

2.3.3.1. Introduction

Urinary ^{51}Cr-EDTA was quantified in the Department of Radiology, Scarborough Hospital. The author worked in conjunction with the departmental staff. The technique adopted was based on that described by Aabakken (Aabakken, 1989). Quantification of urinary ^{51}Cr-EDTA is a relatively straightforward procedure. The main variable which must be taken into account is radioactive decay, the half-life of ^{51}Cr-EDTA being 27.7 days (Firestone *et al*, 1996). If urine samples are counted along with an aliquot of the oral test solution, the effect of decay can be automatically corrected for, as decay will affect both equally. This allows samples to be counted up to four weeks following administration of the test, and enables accurate calculation of fractional excretion. In contrast to the sugar tests of permeability, the exact amount of ^{51}Cr-EDTA administered and the exact concentration in the urine were not important, since the counting method itself provided an accurate estimate of fractional excretion.

Initial experiments to investigate the performance of the gamma counter were performed using a technetium isotope (technetium 99m), as this was readily available from the Department of Radiology, Scarborough Hospital.

2.3.3.2. Reagents

^{51}Cr-EDTA was purchased from Nycomed Amersham plc (Little Chalfont, Buckinghamshire, UK) as vials designed for intravenous injection. Each 10ml vial contained 37 MBq of activity at reference, and comprised chromium edetate (0.64g/L), disodium edetate (1.9g/L) and benzyl alcohol (10g/L). Technetium 99m, as sodium pertechnetate was manufactured on-site using an MCC 20 generator (Nycomed Amersham).

2.3.3.3. Instrumentation

The pulse height analyser used was a Counter Ratemeter MS310 (J&P Engineering, Reading, UK). The scintillation detector consisted of a cuboidal well (dimensions 11 x 11 x 13cm) surrounded by 2.5cm lead shield. This neatly accommodated the 500ml screw-top pots used for urine samples (Henleys Medical, Welwyn Garden City, Herts, UK). The scintillation detector was set into one wall of the well, and comprised a 5 x 5cm cylindrical sodium iodide crystal and a photomultiplier tube. The high tension (HT) voltage for the photomultiplier was generated by the Counter Ratemeter.

2.3.3.4. Experimental Technique

All counting was performed after a one hour "warm up" period. In every case background radiation was first determined by counting a 400ml water "blank," and the background count rate subtracted from that of subsequent analytical samples.

Optimisation of HT Voltage

A technetium 99m standard with nominal activity 100 kBq in 10ml was prepared and placed in the counting well. The energy acceptance window was set to 140 KeV, with a width of 10%. The HT voltage was initially set to 650V, and counting performed for 10 seconds. The HT was increased in 10V increments, counting for 10s at each new voltage. When the count rate began to rise, the HT was increased in 2V increments until a peak was reached and counts fell to baseline levels. The HT was then again increased in 10V increments, to a maximum of 1,000V. When setting each new HT level, the dial was increased from zero, in order to minimize the effect of mechanical "backlash."

The results of this experiment are shown in Figure 21. It can be seen that the optimal HT setting was 808V for 99m technetium. This provided a rough estimate of the optimal HT voltage for ^{51}Cr-EDTA; more precise optimisation was performed daily around the 808V value.

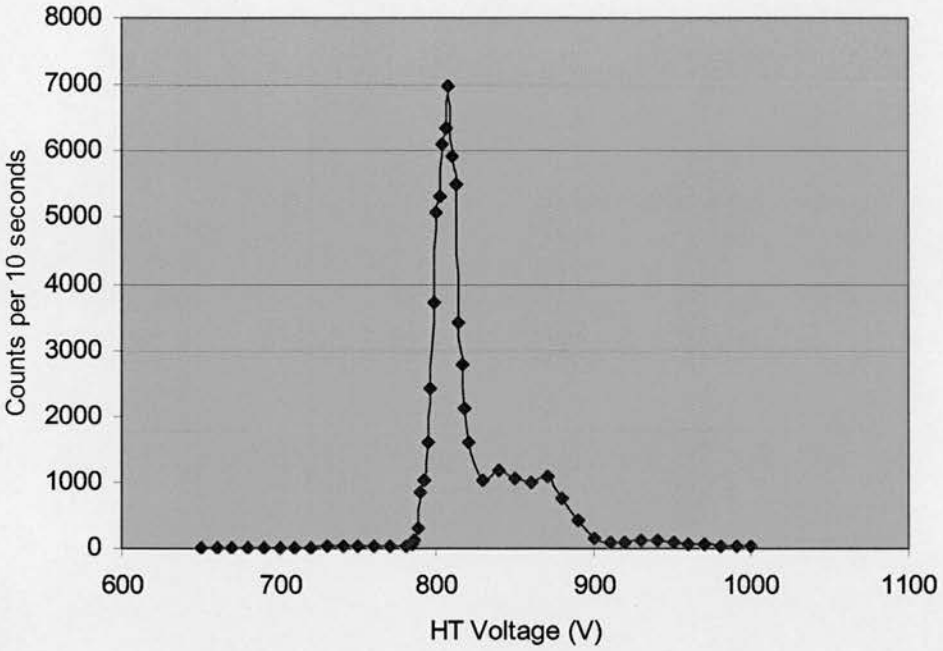


Figure 21. Optimisation of HT Voltage.
This experiment was performed with a technetium 99m standard, and an energy acceptance window of 10%, centered on 140KeV.

Optimisation of Energy Acceptance Window for ^{51}Cr-EDTA

An experiment was then performed to optimize the energy acceptance window for ^{51}Cr-EDTA. A 400ml standard (approximate activity 0.74 kBq/ml) was made up and placed in the counting well in a screw topped container. The HT voltage was set to 808V, and the energy acceptance window width set to 5%. The window centre was initially set to 100 KeV, and counting performed for 15 seconds. The window centre was then increased in 10 KeV increments, counting for 15 seconds at each new setting. When the

count rate began to increase the window was increased in five KeV increments, and around the peak at one KeV increments. The maximum level studied was 400 KeV.

The results of this experiment are shown in Figure 22. It can be seen that the optimal energy acceptance window was centred on 323 KeV, which is in keeping with published gamma emission data for ^{51}Cr (Firestone *et al*, 1996).

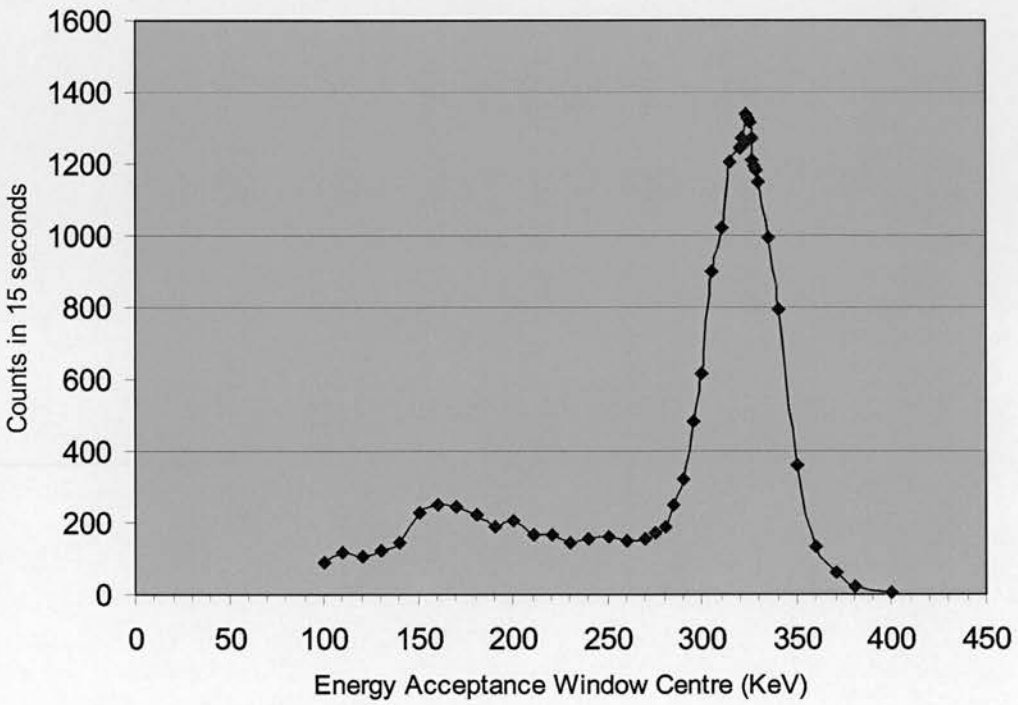


Figure 22. Optimisation of the energy acceptance window for ^{51}Cr-EDTA.
Window width was set to 5%. The peak occurred at 323 KeV.

Linearity of Response

A further experiment was performed in order to ensure linearity of response across the range of activities expected in patients' urine. A Tc99m standard was made up with a nominal activity of 600 kBq in 10ml. Dilution was performed by taking 1ml from this sample and making it up to 10ml. Serial 1 in 10 dilutions were performed in an identical manner until six standards (each with a volume of 9ml) were obtained. These were then counted with the HT set to 808 V and an energy acceptance window of 20%, centered on 140 KeV. Each sample was counted for at least 1,000 counts, and the count rate (counts per second, cps) calculated. A calibration curve of nominal activity against count rate was plotted, and linear regression performed. Count rates were corrected for the decay of Tc99m over the counting period.

The results are shown in Figure 23. It can be seen that the response of the counting system was linear over the activity range 0.0055 kBq to 550 kBq. The regression coefficient (R^2) was 0.999.

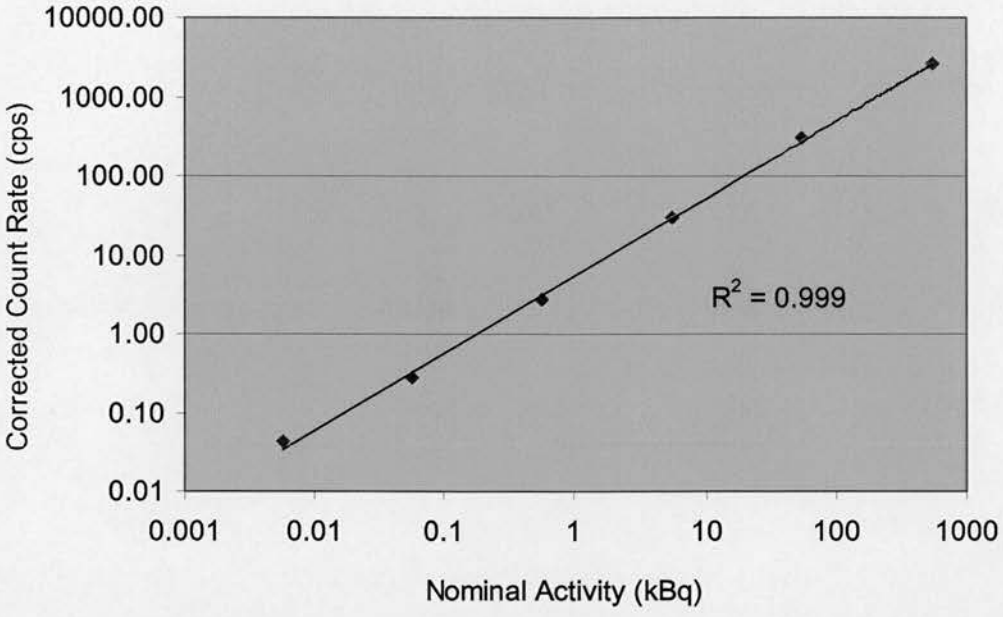


Figure 23. Calibration curve for Tc99m.
Note that a logarithmic scale has been used for both ordinate and abscissa.

Preparation of ^{51}Cr-EDTA Standard

In order to correct for radioactive decay, urine from each subject was assayed along with a standard prepared from the same stock solution as the subject's dose. A 1ml aliquot of a 2.5ml subject dose was diluted to 1L with water in a volumetric flask. Approximately 400ml was decanted into a sample pot, and the exact volume determined by weight, assuming a weight to volume ratio of 1.00. At reference date the approximate activity of the standard solution was 0.74kBq/ml.

Analysis of Subjects' Urine

Each day that analysis of urine samples was to be performed the equipment was switched on and left to "warm up" for at least one hour. Approximately 400ml of the appropriate standard was placed in the counting well. Optimisation of the HT was performed, with an energy acceptance window of 25%, centered on 323 KeV. In view of the results shown in Figure 17, the HT voltage was increased in 1V increments from 790V until the optimal value had been reached. Throughout the period of data collection (approximately one year), the optimal HT remained between 808 and 810V.

Once the optimal HT had been established, a pot containing 400ml of tap water was counted for 1,000 seconds as a measure of background radiation, and the count rate subtracted from all subsequent results. The standard and patient samples were then counted for 1,000 seconds. If the count was less than 10,000 the time was extended appropriately. The count rates for urine samples and standards were corrected for background radiation, and the corrected rates standardised according to the formula:

Standardised Count Rate for 1,000ml =

Corrected Count Rate of Sample (cps) x (1,000 / Sample Volume (ml))

The standardised count rates for 1,000ml of patient urine and 1,000ml of standard were used to calculate percentage excretion according to the following formula:

Urinary Excretion (%) =

$$\frac{\textbf{[(count rate (cps) for 1,000ml urine) x (24-hoururine volume (ml))]}}{\textbf{[(count rate (cps) for 1,000ml standard) x 2.5)]}}$$

cps = counts per second

Note: the figure of 2.5 in the denominator results from the fact that the initial standard dilution was made from a 1ml aliquot of a 2.5ml subject dose.

Variability of the Method

^{51}Cr-EDTA was added to 400ml water to make up four standards with activities approximating that of the urine of patients who had excreted 1%, 2%, 4% and 8% of an oral dose (assuming a hypothetical urine volume of 1600ml). Within-batch variability was assessed by counting each standard 15 times. The results of this experiment are shown in Table 6. The within-batch coefficient of variation was between one and two percent. Between-batch variability was assessed by counting urine samples from control subjects on two separate occasions, one week apart. The coefficient of variation was calculated using a one-way analysis of variance technique (Bland, 2000). The between-batch coefficient of variation was 4.54% in the 19 samples analysed.

Table 6. Within-batch coefficient of variation for ^{51}Cr-EDTA standards

Activity per 400ml (kBq)	Hypothetical Excretion (%)	Number of Counts	Coefficient of Variation (%)
37	8	15	1.22
18.5	4	15	1.17
9.25	2	15	0.91
4.625	1	15	2.24

(Hypothetical excretion values assume a urinary volume of 1600ml and an oral dose of 1.85MBq)

2.3.4. Administration of the ^{51}Cr -EDTA Test

2.3.4.1. Preparation of the Test Solution

A license for the use of ^{51}Cr-EDTA was granted by the Administration of Radioactive Substances Advisory Committee (ARSAC) (Appendix 3). ^{51}Cr-EDTA was purchased from Nycomed Amersham plc (Little Chalfont, Buckinghamshire, UK) as vials designed for intravenous injection. Each 10ml vial contained 37 MBq of activity at reference, and comprised chromium edetate (0.64g/L), disodium edetate (1.9g/L) and benzyl alcohol (10g/L). Preparation of subject doses was performed by the staff of Scarborough Hospital Pharmacy Department. The contents of each 10ml vial were drawn up with 40ml of sterile water in a sterile 50ml syringe to form a stock solution with nominal activity of 0.74MBq/ml. A 2.5 ml aliquot was drawn off for each patient using a 3ml disposable syringe (BD Plastipak™, BD Medical, Franklin Lakes, New Jersey, USA). The accuracy of these syringes was tested by drawing up 2.5ml of deionised water and dispensing it onto an electronic balance. The mean weight of water dispensed over 50 consecutive attempts was 2.491g; the coefficient of variation was 0.699%.

Syringes containing subject doses were stored behind lead, and the contents of one syringe (approximately 1.85 MBq) discharged into a cupful of water (120ml) immediately prior to administering the permeability test. As the half-life of ^{51}Cr-EDTA is 27.7 days, a fresh batch was made up once a month and any unused doses discarded 28 days after the reference date.

2.3.4.2. Osmolality of the Test Solution

Osmolality was measured by analyzing an aliquot of the oral test solution in an osmometer (Advanced™ Micro-Osmometer, Model 3MO Plus, Vitech Scientific Ltd, Partridge Green, West Sussex, UK). Measurement was performed by Scarborough Hospital Clinical Biochemistry department, using a depression of freezing point technique (Kaplan *et al*, 1996). The mean osmolality of three repeat samples was seven mOsmol/kg (range 6-8 mOsmol/kg).

2.3.4.3. Administration of the Test Solution

Subjects were asked to refrain from alcohol for 24 hours prior to the test, and to fast from midnight. The ^{51}Cr-EDTA permeability tests were administered by the author between 0830 and 0930 hrs the following morning. Subjects were witnessed drinking the entire solution. After ingesting the test solution subjects were allowed to drink water for the first two hours and to eat and drink freely following this. Subjects were asked to refrain from alcohol until they had completed a 24-hour urine collection.

2.3.4.4. Urine Collection

Urine was collected in a single container, with 1ml 10% sodium merthiolate as a preservative. Subjects were asked to void urine immediately before the permeability test, and to collect all urine passed during the subsequent 24 hours. At the end of the 24-hour period patients were asked to void into the container for the last time, and to return the urine container to Scarborough Hospital.

2.3.4.5. Sample Preparation

Subjects were asked to return the urine container to the hospital within two days of completing the permeability test. Containers were weighed on an electronic flat-pan balance (LS 5000, Ohaus, Pine Brook, NJ, USA), the weight of an empty container + preservative subtracted, and the corrected weight used as a measure of volume, assuming a weight to volume ratio of 1.00. Approximately 400ml of urine was decanted into a sample pot (Henleys Medical, Welwyn Garden City, Herts, UK) and the exact volume again determined by weight. Activity was measured using a gamma counter and corrected for radioactive decay using a standard (section 2.3.34).

2.4. Discussion of the Methods

2.4.1. Development of the Triple Sugar Test

The aim of this study was to investigate the use of a triple sugar test of intestinal permeability as a surrogate marker of gut barrier function in surgical patients. At the time the study was performed, sucralose was not in use as a permeability probe in the UK. The first stage of this thesis project was therefore to develop a suitable method for the quantification of sucralose in urine.

When deciding which analytical technique to employ, it was decided to adopt HPLC with amperometric detection, as this technique had been successfully used by Medding's group in Canada (Meddings *et al*, 1998, Smecuol *et al*, 2001). In addition, HPLC with amperometric detection was a well-established technique for the quantification of other urinary sugar probes such as lactulose, mannitol and L-rhamnose (Fleming *et al*, 1990, Fleming *et al*, 1993, Sorensen *et al*, 1993).

Prior to analysis of urinary sucralose, with its attendant complexities, it was attempted to quantify sucralose in aqueous solution. The equipment and conditions employed were as similar to those described by Meddings as was practicable. Initial analysis of mannitol, L-rhamnose and glucose confirmed that the chromatography system and pulsed amperometric detector (PAD) were in working order and capable of detecting sugars in

concentrations appropriate to the level of urinary sucralose expected during the triple sugar test (Fleming *et al*, 1990, Smecuol *et al*, 2001).

The published method for sucralose analysis was then attempted. This involved changing the mobile phase, the column, and adding a post-column flow of sodium hydroxide. When these changes had been made and sucralose was injected no peaks were identified. This implied that either sucralose was retained on the column, or it was eluted but not detected. It was decided to investigate whether or not sucralose was detected by the PAD system.

In order to ensure that sucralose was reaching the detector the analytical column was removed and the analytes injected directly onto the detector. The variables in this experiment were thus reduced to the efficiency of the detector, the effects of post-column addition, and the interaction between sucralose and the detector. The detector and post-column addition apparatus were shown to be in working order when mannitol, L-rhamnose and glucose were analysed – a large spike was seen shortly after injection. However, when sucralose was injected the baseline remained flat.

The results of these experiments suggested that sucralose was not detected using a pulsed amperometric detection system which worked well with other sugars. This negative result seemed independent of chromatographic conditions or the use of post-column addition, albeit this was a somewhat imperfect system. The chlorinated structure of sucralose was likely to have affected its electrochemical properties, and may have been

responsible for the lack of detection. At this point the technique of HPLC-PAD was abandoned. Following discussion with the Sucralose Research Arm of Tate & Lyle UK (Mary Quinlan, personal communication), it was decided to attempt HPLC with refractive index detection (HPLC-RI).

Initial studies with the HPLC-RI system demonstrated that sucralose was detected in concentrations approaching those expected in subjects' urine. An internal standard was included in the method, although not described by Tate & Lyle. This was deemed good practice, providing early warning of fluctuation in injection volumes or detector sensitivity. The analysis of 60 standards of sucralose in urine demonstrated that the system was both accurate (mean recovery 103.1%) and precise (coefficient of variation 6.07%). As might be expected, the coefficient of variation was inversely related to the concentration of sucralose in the standard (4.47% at a concentration of 100mg/L *versus* 7.59% at a concentration of 25mg/L). The limit of detection, as calculated using a signal to noise ratio, was 11mg/L, which is almost identical to published data (Kobayashi *et al*, 2001). This necessitated an oral dose of 5g of sucralose, which was 2.5 times that given in previous clinical studies employing HPLC-PAD or GC-FID methods of analysis (Enns *et al*, 1997, Smecuol *et al*, 2001, Suenaert *et al*, 2000).

Urinary lactulose and L-rhamnose levels were quantified by HPLC-PAD, as described previously (Fleming *et al*, 1990, Fleming *et al*, 1993, Sorensen *et al*, 1993). The method of HPLC-PAD has been shown to be both accurate and precise, and is over 10 times more sensitive than the HPLC-RI method used to quantify urinary sucralose. Analytical

recovery (mean ± SD) of urinary lactulose using this method in two studies was 97.6 ± 6.8% at a concentration of 6mg/L (Fleming *et al*, 1993)and 100 ± 6.1% at a concentration of 2mg/L (Sorensen *et al*, 1993). In the current study it was demonstrated that sucralose did not interfere with the analysis of lactulose or L-rhamnose (section 2.3.1.4).

The "clinical" methods relating to the preparation and administration of the triple sugar test were standardised as far as possible. Dietary restrictions, timing of the tests and the method of urine collection were consistent between individuals. All permeability tests were performed by the author, and the ingestion of every test solution witnessed.

The eight hour fast was designed to ensure that subjects did not have food residue in the small bowel prior to the test, in an attempt to standardize intra-luminal intestinal conditions. It was recognized that the majority of intraluminal fluid was likely to have been secreted by the gastrointestinal tract itself, and that this was likely to have varied between subjects. This may have resulted in differences in osmotic gradients of test probes, which may have influenced permeability results. However, an overnight fast standardised basal conditions as far as possible, and was in keeping with most studies of intestinal permeability.

Following ingestion of the triple sugar test, patients were only allowed to drink water for the first five hours, and to eat and drink freely following this. This was something of a compromise between the ideal and the practical. Ideally, subjects would not consume anything by mouth over the entire 24-hour period, in order to standardize intraluminal

osmotic gradient and transit. However, this was clearly neither ethical nor practicable. The practice of five hours restriction to water had become established in the author's institution for tests of small bowel permeability (lactulose/rhamnose). The aim of this practice was to prevent ingested food residue from interfering with probe absorption through mechanical or osmotic effects, and to ensure that traces of dietary lactulose or rhamnose would not confound urinary analysis of these sugars.

In reality there is very little lactulose, L-rhamnose or sucralose in the British diet, and these concerns were probably unfounded. As an alternative, many authors have insisted on a 2-hour complete fast following ingestion of test subtances, followed by free diet. The rationale behind the figure of two hours is that this allows time for the test probes to reach the caecum (Table 5). However, many people do not tolerate complete fasting very well. In the current study many of the ileostomists expressed concern about an overnight fast, as they tended to drink more than average in order to keep up with stoma losses. It was decided that prolongation of fluid restriction, even by two hours, was not justified in an investigation of whole-intestinal permeability. Diet was therefore restricted to water for five hours, which enabled comparison of results with those of earlier studies of small intestinal permeability in the author's institution.

In addition to fasting overnight, all subjects were asked to refrain from alcohol for 24 hours prior to ingestion of the test drink and for the duration of urine collection. Patients were asked to omit NSAID's for 48 hours prior to the test and for the duration of urine collection. Alcohol and NSAID's have been shown to influence the intestinal absorption

of a variety of permeability probes (Aabakken, 1989, Jenkins *et al*, 1991, Krugliak *et al*, 1990, Smecuol *et al*, 2001)

Many investigators have added osmotic fillers such as glycerol to permeability test solutions in order to "stress" the intestine. This procedure has been reported to increase the specificity of dual sugar tests in detecting intestinal damage caused by disorders such as coeliac disease (section 1.5.1). In the current study an approximately iso-osmolar solution was used for several reasons. Firstly, the triple sugar test was designed as a measure of whole gut, rather than purely small-intestinal permeability. Whilst a hyperosmolar test solution might apply some "stress" to the stomach and proximal small intestine, this effect is likely to be reduced as the relatively small volume of test solution (150ml) mixes with the considerably larger volumes of secretions present in the small intestine, resulting in a negligible osmotic effect on the colon.

Secondly, the practice of adding osmotically active substances to test solutions has resulted in somewhat unpredictable alterations in permeability. Rather than increasing permeability, a small increase in osmolality has, in some instances, been shown to reduce probe excretion. This has been postulated to be due to fluid shift and reduced intestinal transit times.

Thirdly, an iso-osmolar solution had been employed in the few published studies where sucralose had been used as a permeability probe. The use of an iso-osmolar solution in the current study facilitated comparison of results with published data.

2.4.2. ^{51}Cr-EDTA Test

The technique for assaying urinary ^{51}Cr-EDTA was relatively straightforward. The series of experiments described in section 2.3.3.4 was designed to optimise the accuracy and precision of the assay technique. Initial experiments confirmed that the energy detection window should be set to 323KeV for ^{51}Cr, and that the response of the detector was linear over a nominal activity range of 0.0055 kBq to 550 kBq (assuming a 24-hour excretion of 2% in 1500ml, an analytical sample of a control subject's urine would be expected to have an approximate activity of 9.9kBq). Counting against a precise dilution of the appropriate oral test solution ensured that an accurate measure of fractional excretion was obtained without the need for measuring absolute concentration, and that this was corrected for the effect of radioactive decay. Standardisation of the analytical sample volume to approximately 400ml ensured that counting geometry was similar for every sample. The between-batch coefficient of variation was 4.5% (section 2.3.3.4), which was an acceptable figure.

Dietary conditions were similar to those employed in the triple sugar test (section 2.4.1). The only exception was that patients were allowed to eat two hours following ingestion of the test solution, as opposed to the five-hour cut-off used in the sugar test. The reason for shortening the "water only" period was that subjects could not ingest ^{51}Cr in their diet, and so could not influence the permeability result by early resumption of normal diet. In addition, most published data relates to recommencement of normal diet two hours

following permeability testing with ^{51}Cr-EDTA (Jenkins *et al*, 1987, Jenkins *et al*, 1988, Peled *et al*, 1985, Pironi *et al*, 1990).

2.4.3. The Lactulose-Hydrogen Breath Test

The lactulose-hydrogen breath test is a well-established method of assessing oro-caecal transit time (section 2.3.2.1). In the current study the test was used in a slightly unconventional manner, in order to give a qualitative assessment of bacterial load in the gastrointestinal tract in healthy volunteers and ileostomists. A hand-held hydrogen meter was used instead of breath sampling and gas-chromatography as it gave instantaneous results and was much easier to use. This method has previously been validated for the purpose of identifying lactase deficiency (Fleming, 1990). Orocaecal transit time (OCTT) was measured as an incidental part of the lactulose-hydrogen breath test in the current study. The median OCTT in healthy volunteers was comparable with that seen in previous studies, which provides validation of the method (Table 5).

Breath hydrogen excretion following lactulose ingestion has not previously been correlated to intestinal bacterial load in healthy volunteers, and so it was not possible to quantitatively relate breath hydrogen concentration to bacterial concentration. However, the test directly measured a product of lactulose fermentation (hydrogen). It should, therefore, have provided a measure of lactulose fermenting capability.

2.4.4. Summary

All three methods developed for the analysis of urinary probe markers (HPLC-RI for sucralose, HPLC-PAD for lactulose and L-rhamnose and radio-counting for ^{51}Cr-EDTA) were both accurate and precise. The inter-assay coefficient of variation of all three methods was of the order of 5%, which is an acceptable figure when compared with the within-group biological variation in permeability (for example, the coefficient of variation of five and 24-hour urinary excretion in control subjects was >30% for all probes studied).

The conditions relating to the preparation and administration of test solutions were standardised as far as possible. Dietary restrictions, timing of the tests and the method of urine collection were consistent between individuals. All permeability tests were performed by the author, and the ingestion of every test solution witnessed.

Part 3: Clinical Studies

3.1. Control Subjects and Ileostomists

3.1.1. Introduction

Following the development of an accurate and precise analytical technique for quantifying sucralose in urine, the triple sugar test was applied to a group of control subjects and a group of healthy patients with ileostomies. This experiment was designed to establish the normal ranges of probe excretion in the author's institution, and to provide information regarding the sites of absorption of sucralose and lactulose along the gastrointestinal tract. For the first time in humans, the hypothesis that urinary sucralose excretion represented colonic permeability was tested. Sucralose excretion was compared to that of ^{51}Cr-EDTA, an established marker of "whole gut" permeability.

3.1.2. Subjects and Methods

3.1.2.1. Control Subjects

Control subjects were healthy members of hospital staff. Subjects with previous gastrointestinal disease or renal impairment were excluded. Twenty-one control subjects (12 females) successfully completed the triple sugar test of intestinal permeability. The median (IQR) age was 46 (33-51) years. All subjects were offered the ^{51}Cr-EDTA test within two weeks of the sugar test. One subject refused, and one subject failed to complete a 24-hour collection, leaving 19 patients with ^{51}Cr-EDTA data.

One control subject was receiving long-term hormone replacement therapy at the time of the test, none of the other subjects required regular medication. No subject had received non-steroidal anti-inflammatory medication (NSAIDs) within 48 hours of permeability testing, or alcohol within 24 hours.

After participating in the triple sugar and ^{51}Cr-EDTA tests of intestinal permeability, six control subjects (2 women) underwent a lactulose-hydrogen breath test, as described in section 2.3.2.3. No subject had received antibiotic therapy in the month prior to the test.

3.1.2.2. Ileostomists

Eighteen subjects (eight women) with ileostomies underwent the triple sugar test. Ten subjects had end-ileostomies following colectomy for ulcerative colitis; the remaining eight subjects had loop ileostomies following excision of rectal cancer. There was no evidence of residual disease in any subject.

The median age of ileostomists was 66 years (IQR 56-70), which was significantly older than control subjects ($p=0.018$, Mann-Whitney U). The median time between formation of ileostomy and the triple sugar test was 20 months (IQR 6-69).

Six ileostomists did not take medication of any description. Of the remaining 12, one took a NSAID (stopped for 48 hours prior to the test), three took diuretics, one took an aminosalicylate and one took oral steroids. No subject had received non-steroidal anti-

inflammatory medication (NSAIDs) within 48 hours of permeability testing, or alcohol within 24 hours.

Sixteen of the 18 ileostomists (6 women) underwent the ^{51}Cr-EDTA test within two weeks of the triple sugar test. Three of the subjects with loop ileostomies (all women) subsequently underwent surgery to reverse their stomas, and were re-tested with the triple sugar test. The median time from reversal of ileostomy to the triple sugar test in these three subjects was six days (range 3-23).

Six of the 18 ileostomists (4 women) subsequently underwent the lactulose-hydrogen breath test. Five subjects had end-ileostomies following colectomy for ulcerative colitis, and one subject had a loop ileostomy following excision of rectal cancer. No subject had received antibiotic therapy in the month prior to the test.

3.1.3. Results

3.1.3.1. Urine Volumes

The median (IQR) five-hour urine volume for control subjects was 452ml (215-708), and for ileostomists 163ml (103-297) (p=0.001, Mann-Whitney U). The median 24-hour urine volumes for control subjects was 1984ml (1400-2504), and for ileostomists 997ml (683-1535) (p=0.007, Mann-Whitney U).

3.1.3.2. Lactulose and L-Rhamnose

Figure 24 demonstrates five and 24-hour lactulose/rhamnose (L/R) excretion ratios. The median five-hour L/R excretion ratio in control subjects was 0.0244 (IQR 0.0216-0.0350). Comparison with Table 1 confirms that this is in keeping with the results of previous studies using lactulose and L-rhamnose as permeability probes in healthy volunteers. Twenty-four hour lactulose excretion (median 0.517% (0.388-0.617)) in the current study was also in keeping with published data (Jenkins *et al*, 1991, Maxton *et al*, 1986). Approximately 40% of 24-hour lactulose excretion occurred in the first five hours (Figure 25). The corresponding figure for L-rhamnose was 86% (Table 11).

The five-hour L/R ratio was similar in controls and ileostomists (0.024 (0.022-0.034) *versus*. 0.025 (0.022-0.035) respectively, p=0.210). However, the 24-hour L/R ratio was significantly higher in controls than ileostomists. It can be seen from Figure 25 that this was due to a two-fold difference in lactulose absorption between control subjects and ileostomists.

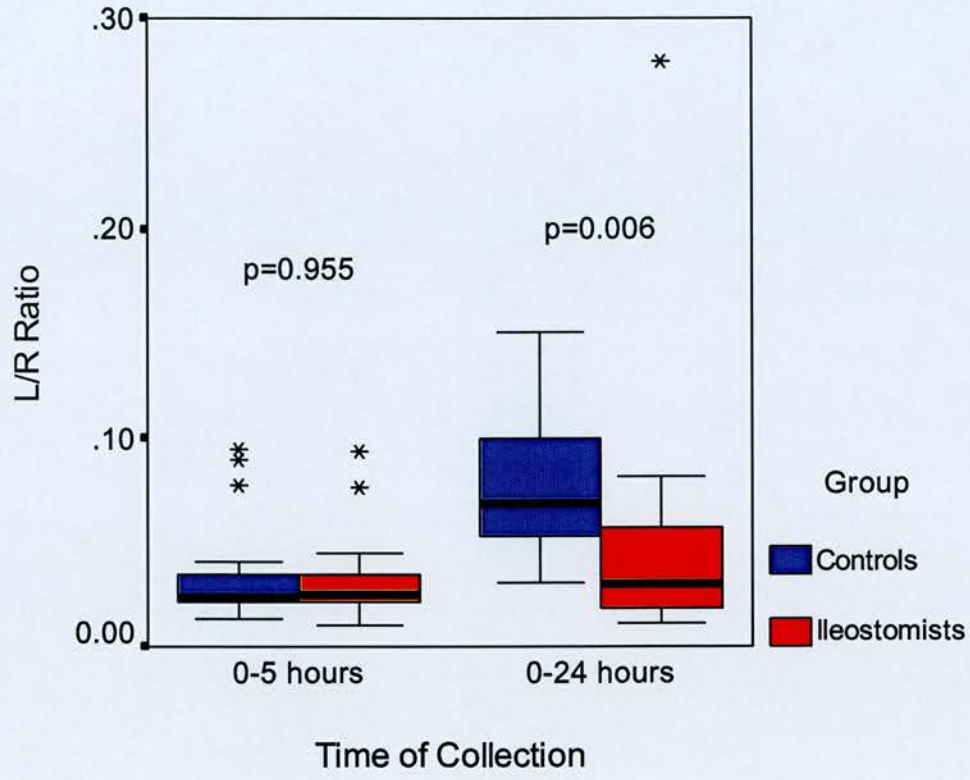


Figure 24. Lactulose/rhamnose excretion ratio

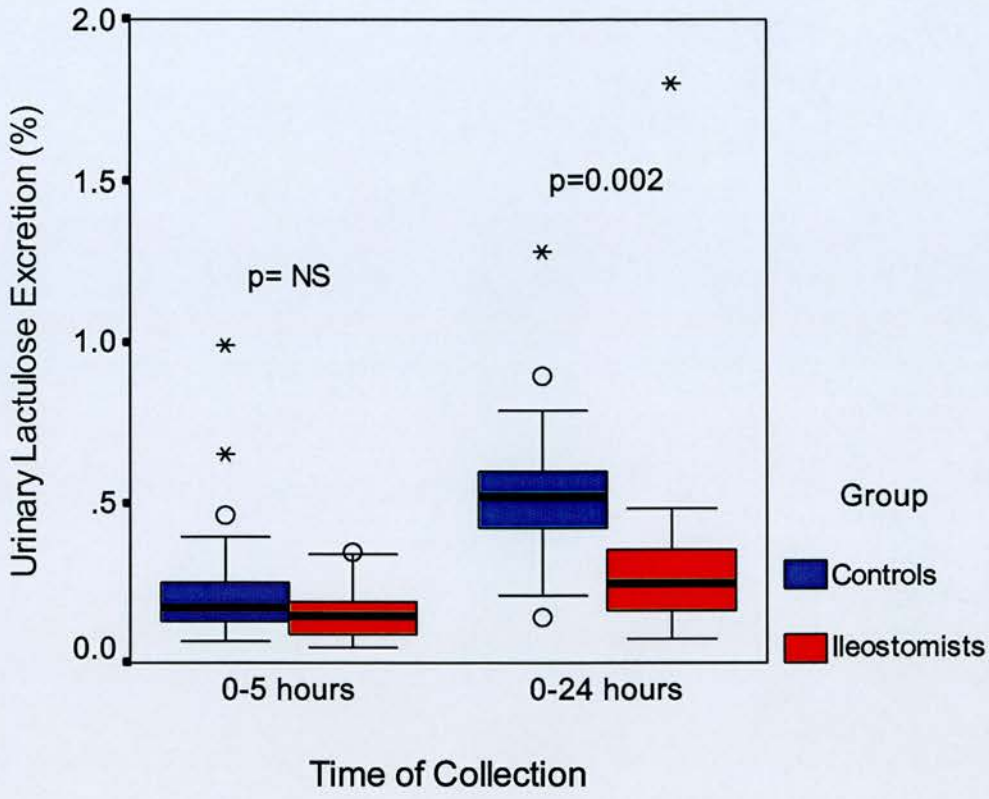


Figure 25. Urinary lactulose excretion

3.1.3.3. Sucralose

Control subjects excreted 1.41% (1.12-1.68) of administered sucralose over 24 hours (Figure 26). Fifty-three percent (IQR 46.6-59.3) of this occurred during the first five hours of collection. Twenty-four hour excretion was slightly lower than that seen in previous studies (Smecuol *et al*, 2001, Suenaert *et al*, 2003).

Sucralose excretion was significantly lower in ileostomists than in control subjects over both five and 24 hours; the difference was more marked over 24 hours (0.96% (0.63-1.24) *versus* 1.41% (1.12-1.68), p=0.003). The proportion of 24-hour sucralose excretion which occurred during the first five hours of collection in ileostomists was 57.5% (IQR 38.3-66.0, p=0.571 compared to controls).

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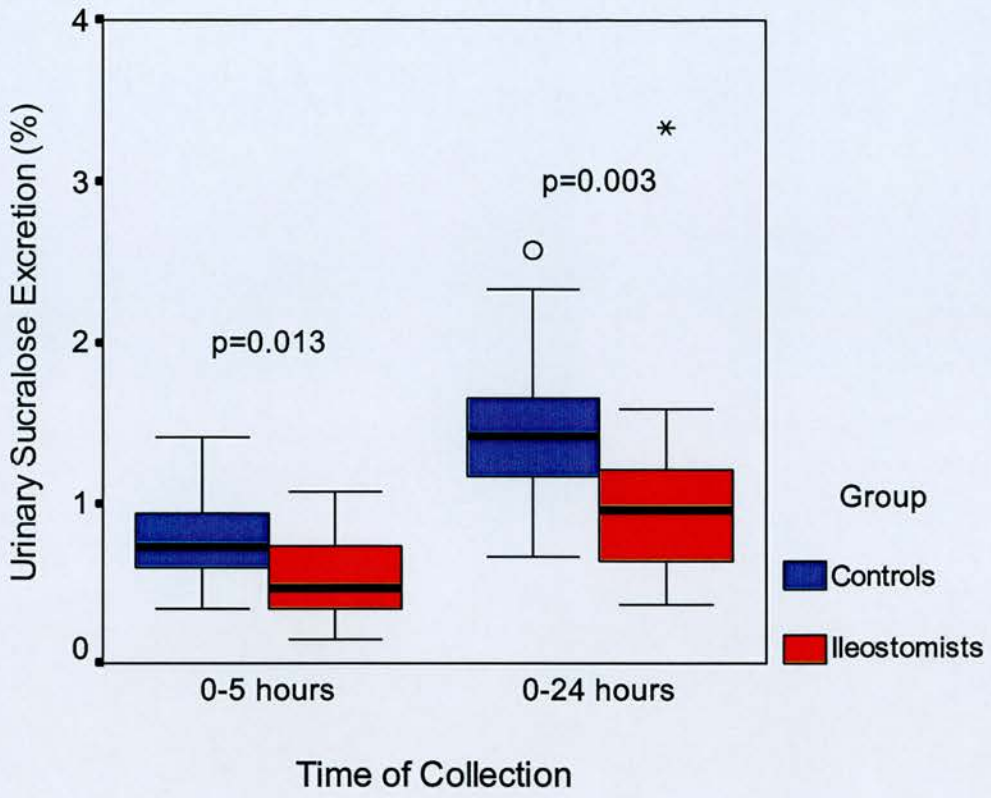

Figure 26. Urinary sucralose excretion

3.1.3.4. ^{51}Cr-EDTA

Control subjects excreted 2.73% (1.94-3.82) of administered ^{51}Cr-EDTA over 24 hours (Figure 27). This figure was slightly higher than that seen in the majority of previous studies (see Table 2), however, it fell within the 95% confidence interval for 24-hour ^{51}Cr-EDTA excretion demonstrated by Aabakken in his seminal methodological study (Aabakken, 1989).

Twenty-four hour urinary ^{51}Cr-EDTA excretion was significantly reduced in ileostomists compared with control subjects, in keeping with sucralose and lactulose excretion. ^{51}Cr-EDTA excretion demonstrated a positive correlation with sucralose excretion, however this was not statistically significant (R=0.264, R^2=0.070, p=0.062).

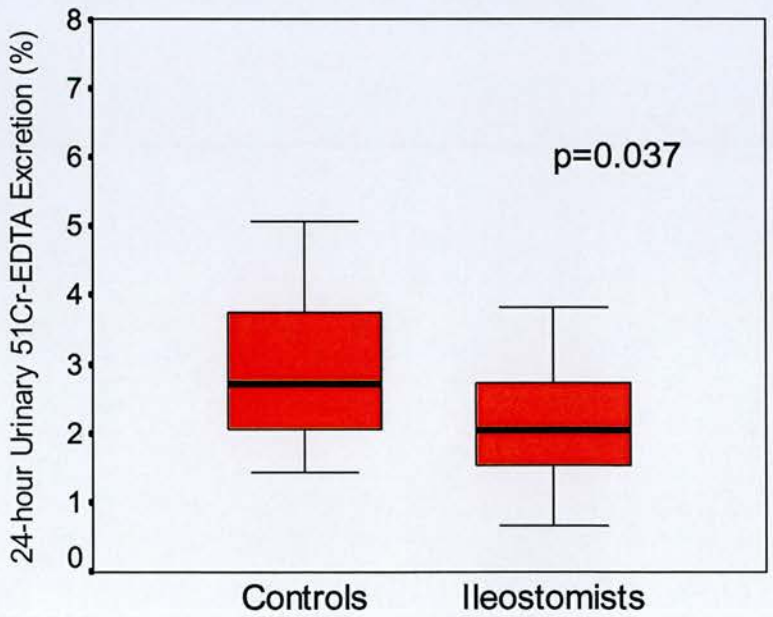

Figure 27. Urinary ^{51}Cr-EDTA Excretion

3.1.3.5. The Triple Sugar Test in Patients Who Underwent Reversal of Ileostomy

The results of the triple sugar test in the three subjects who underwent reversal of ileostomy are shown in Figure 28. Pre-reversal, excretion of the three sugar probes was representative of that seen in the larger group of 18 ileostomists. Following reversal, 24-hour excretion of sucralose and lactulose increased to levels similar to those seen in control subjects. These increases were not statistically significant, due to the small sample size (n=3). The five-hour L/R ratio was similar pre- and post-reversal, which also mirrors the results of testing control subjects and ileostomists (section 3.1.3.2). These results confirm that the differences in probe excretion between control subjects and ileostomists described above were due to the amount and type of functioning intestine, and not to confounding factors such as chronic adaptation of small intestinal absorption in ileostomists.

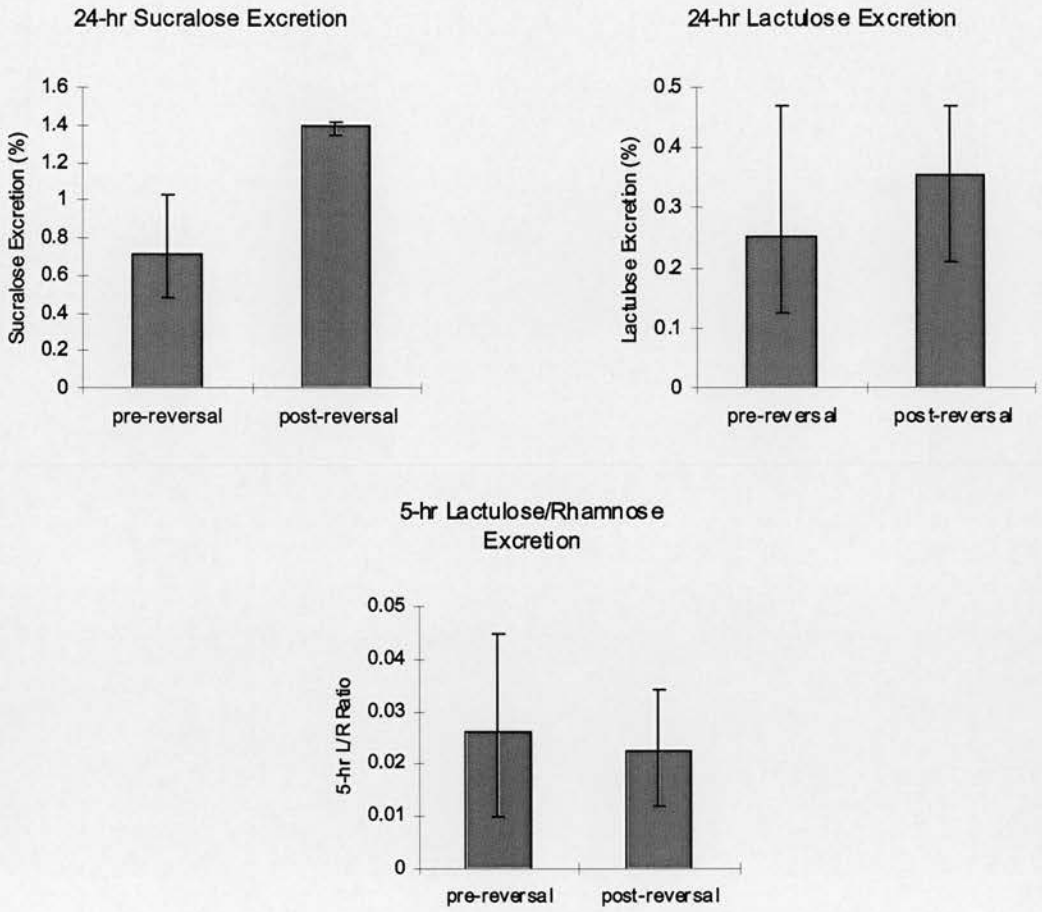

Figure 28. Results of the triple sugar test in three ileostomists pre- and post-reversal.

Bars represent mean value, error bars represent the range. There were no statistically significant differences between pre- and post-reversal tests ($p>0.05$, paired t-test).

3.1.3.6. Lactulose-Hydrogen Breath Tests

End-tidal breath hydrogen concentrations for control subjects and ileostomists are shown in Figures 29 and 30, respectively. All six control subjects demonstrated a significant and sustained rise in breath hydrogen excretion following the administration of lactulose. In contrast, none of the ileostomists demonstrated a significant rise (p=0.002, Fisher's Exact Test). Median oro-caecal transit time (defined as the time breath hydrogen first reached >20ppm above baseline) was 75 minutes (range 60-165) in control subjects, which is in keeping with published data (Table 5).

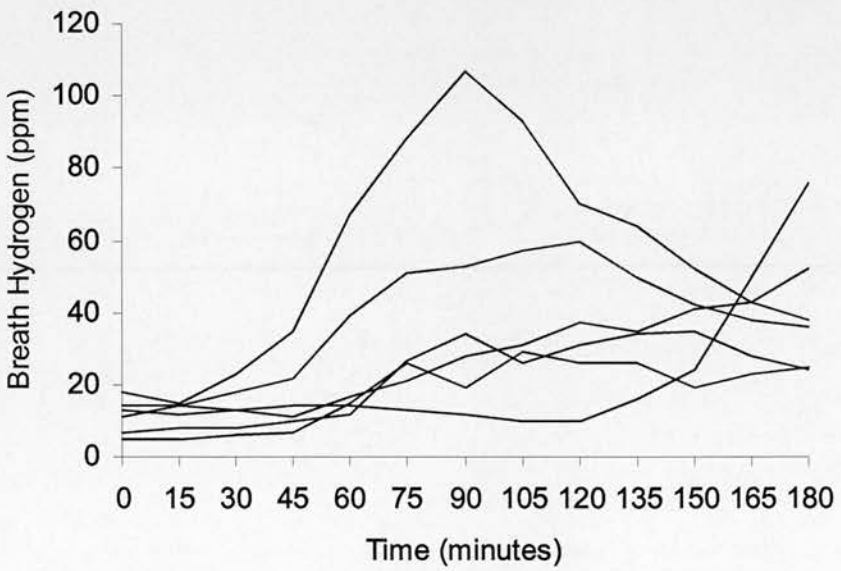

Figure 29. End-tidal breath hydrogen levels in six control subjects
All six subjects demonstrated a rise of >20ppm above baseline levels following lactulose ingestion

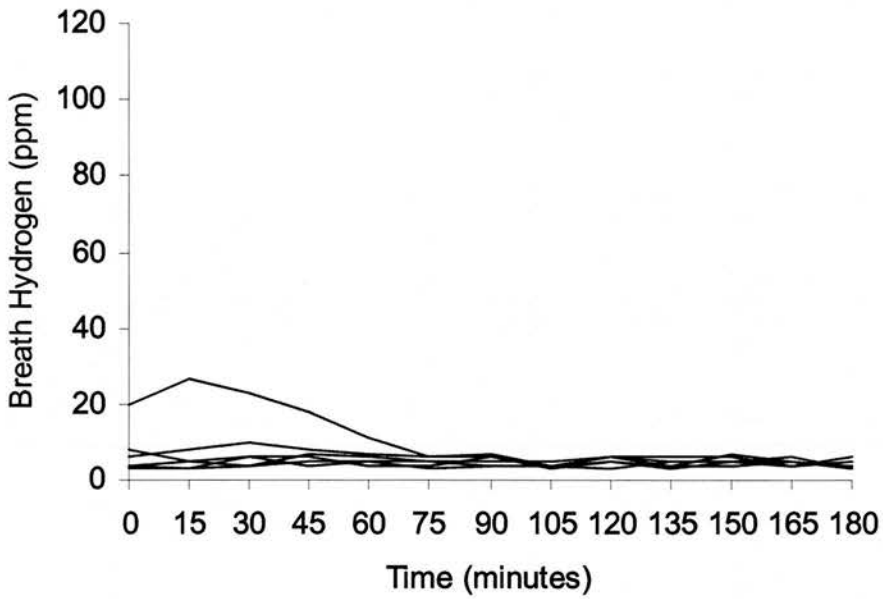

Figure 30. End-tidal breath hydrogen levels in six ileostomists
No subject demonstrated a rise of >20ppm above baseline levels following lactulose ingestion

3.1.4. Discussion

3.1.4.1. Permeability Results in Control Subjects

The median five-hour lactulose excretion and L/R ratio in control subjects were very similar to the results of previous dual-sugar probe studies in healthy volunteers. This validates the clinical and laboratory methods used in the current study, and supports the assumption that the group of 21 control subjects was a representative sample of healthy individuals.

Twenty-four hour lactulose excretion was also similar to that seen in previous studies. However, the time-course of lactulose excretion in this study differed from that seen previously. In the current study, approximately 40% of 24-hour lactulose excretion occurred during the first five hours of collection (Table 11). In previous studies in healthy volunteers, this figure has been of the order of 65-70% (Elia *et al*, 1987, Maxton *et al*, 1986). As these prior studies involved both iso-osmolar and hyperosmolar solutions, composition of the test drink is unlikely to be the cause of this discrepancy. One possible explanation is that the number or composition of colonic bacteria in control subjects in the current study differed from that of healthy volunteers in previous studies. A difference in colonic microflora might result in a difference in lactulose fermenting capability, and so affect 24-hour lactulose excretion. This raises the possibility that alterations in colonic microflora might be a confounding factor in the interpretation of 24-hour lactulose excretion.

The median 24-hour sucralose excretion in control subjects was 1.41% (mean 1.47%). This was slightly lower than that seen in previous clinical studies (Enns *et al*, 1997, Smecuol *et al*, 2001, Suenaert *et al*, 2000). This minor discrepancy implies a difference in probe absorption, intestinal transit or renal excretion between subjects in the current study and those investigated previously. This is not surprising. The normal range of intestinal permeability to any probe varies considerably from unit to unit, as demonstrated with regard to lactulose in Table 1. This occurs as a result of differences in test solution volume, composition and osmolality. In addition, there are often slight differences in dietary restrictions. It is rare to find two units using an identical analytical technique, which adds further variability to permeability testing. Furthermore, intestinal permeability exhibits significant biological variability, which is likely to result in differences in the "normal" range of permeability reported by different authors, as this is usually assessed by testing relatively small groups of control subjects.

In previous studies employing sucralose as a permeability probe, sucrose was added to test solutions, rendering them hyperosmolar (Smecuol *et al*, 2001). This was not part of the current triple sugar test, which employed an iso-osmolar test solution. A dose of 2g sucralose was administered previously. Due to the limitations of HPLC-RI, a higher dose (5g) was employed in the current study. In addition, subjects in the current study were allowed to drink water immediately following ingestion of the test solution, whereas a variable period of fasting had been employed previously.

Any one (or a combination) of these factors could explain the difference in sucralose excretion between volunteers in this and previous studies.

Median 24-hour ^{51}Cr-EDTA excretion was 2.73%. It can be seen from Table 2 that this was slightly higher than that seen in previous studies, but there has been significant variation in results from different units. One of the most rigorous descriptions of the ^{51}Cr-EDTA permeability test was that performed by Aabakken (Aabakken, 1989). The method for analysis of ^{51}Cr-EDTA in the current study was similar; in particular a large volume count chamber was used (400ml in the current study, 1,000ml in Aabakken's study *versus* 0.5-5ml in other studies (Jenkins *et al*, 1991, Maxton *et al*, 1986, Pironi *et al*, 1990). The mean 24-hour excretion of ^{51}Cr-EDTA in Aabakken's study was 2.45% (CI 95 = 2.11-2.86%).

The similarity between the results of the current study and those of Aabakken's study suggests sound methodology. It also confirms that the group of control subjects in the current study was a representative sample of the general population in terms of whole-intestinal permeability.

Summary

The results of both the triple sugar and ^{51}Cr-EDTA tests in control subjects were similar to those seen in previous studies employing healthy volunteers. This gives confidence in the methods used, and confirms that the 21 controls used in the current study were a representative sample of the general population, in terms of intestinal permeability. The

fact that the current results differ slightly from previous studies emphasises the need for each unit to define a normal range of urinary probe excretion using a standardised test protocol. It also highlights the importance of not deviating from that protocol at a later date. It is inappropriate to compare results of permeability testing to those of historical control subjects, or those from other units. The most significant difference between control subjects in this and previous studies was in the time-course of lactulose excretion, which may reflect differences in colonic microflora between study groups.

3.1.4.2. Permeability Results in Ileostomists

The purpose of performing permeability tests in subjects with ileostomies was to estimate the relative contributions of the small and large intestine to probe absorption. If control subjects are assumed to have normal small and large intestinal function, and ileostomists normal small intestinal function, then 24-hour urinary probe excretion in ileostomists represents small intestinal permeability, whereas 24-hour probe excretion in control subjects represents "whole gut" permeability. The subtraction of median 24-hour excretion in ileostomists from median 24-hour excretion in control subjects should result in an approximation of colonic probe absorption in healthy subjects.

It could, however, be argued that the remaining small intestine was not "normal" in the ileostomists due to residual disease, alteration in absorptive function, abnormal transit, or bacterial overgrowth. Each of these possibilities is considered unlikely, for the following reasons. The 18 ileostomists were selected on the basis of an underlying diagnosis of ulcerative colitis or rectal cancer, with normal small bowel. No subject had clinical

evidence of gastrointestinal disease at the time of study. Furthermore, five-hour lactulose and L/R excretion ratios, the traditional measures of small intestinal permeability, were similar between ileostomists and controls. This suggests that small intestinal para-cellular permeability and transit were not significantly affected by the formation of an ileostomy, a finding which is in keeping with published data (Elia *et al*, 1987, Jenkins *et al*, 1991). The reduced urine volumes in ileostomists did not affect absolute or fractional five-hour lactulose excretion, suggesting that a difference in renal blood flow between the two groups was not a significant confounding factor. Due to practical considerations, the lactulose-hydrogen breath test was performed in a random selection of six ileostomists and six control subjects. This test provided a direct measure of bacterial fermentation in the intestinal tract. In the sample studied, no ileostomist demonstrated measurable fermentation of lactulose, suggesting that bacterial colonization of the small intestine did not occur to a significant degree.

It was concluded, therefore, that small intestinal function of ilestomists and control subjects was similar in terms of transit, permeability and bacterial load. Differences in 24-hour permeability between the two groups were therefore likely to be a consequence of colonic permeability.

There was no difference in five-hour lactulose excretion, or the five-hour L/R ratio between ileostomists and control subjects, confirming that small intestinal permeability was similar between the two groups. Lactulose excretion over 24 hours in control subjects (median 0.517%) was greater than that in ileostomists (median 0.248%), by a factor of 2.08. This finding is at odds with the results of previous studies in a small number of ileostomists, which demonstrated no difference in 24-hour lactulose excretion between ileostomists and control subjects (Elia *et al*, 1987, Jenkins *et al*, 1991). In the absence of a difference in small intestinal permeability, it can be assumed that the difference in 24-hour excretion between control subjects and ileostomists was a consequence of colonic absorption. The results of this study therefore imply that approximately 50% of lactulose absorption occurred in the colon of control subjects. This figure might be expected to vary depending on the lactulose load presented to the colon, and the capacity of colonic bacteria to ferment lactulose.

The unexpected finding of colonic lactulose absorption has important implications for the use of dual and triple sugar permeability tests. In particular, the subtraction of 24-hour lactulose absorption from that of a non-fermented probe (such as sucralose or ^{51}Cr-EDTA) is unlikely to result in a meaningful estimation of colonic permeability, as lactulose itself appears to be absorbed in the colon.

Twenty-four hour excretion of sucralose was higher in control subjects than ileostomists, by a factor of 1.48. Assuming that small intestinal sucralose absorption was similar in ileostomists and controls, the amount of sucralose absorbed in the colon of control

subjects could be approximated by subtracting the 24-hour sucralose excretion in ileostomists (median 0.955%) from the 24-hour excretion in controls (median 1.41%). This gives a hypothetical colonic absorption of 0.455%. This figure represents 32% of the 24-hour sucralose excretion in control subjects, implying that approximately a third of sucralose absorption occurred in the colon in healthy controls.

As sucralose is not a substrate for colonic bacterial fermentation, it is not surprising that a significant proportion of 24-hour excretion resulted from colonic absorption. This was the premise for using sucralose to measure colonic permeability in the current study, and in previous studies of conditions such as NSAID-induced intestinal damage (Smecuol *et al*, 2001), and following the application of nicotine patches (Jenkins *et al*, 1991).

The results of the triple sugar test in control subjects and subjects with ileostomies confirm that sucralose is absorbed throughout the length of the gastrointestinal tract. As such, it is impossible to differentiate small intestinal from colonic permeation when interpreting a single 24-hour urinary excretion figure. Rather, 24-hour sucralose excretion must represent "whole gut" permeability.

It is of note that, in the ileostomists, almost as much sucralose was excreted in the latter part of the collection (5-24 hours) as was excreted in the first five hours. As small-intestinal transit time is considerably less than five hours in the vast majority of subjects (Jorge *et al*, 1994, Ladas *et al*, 1989), this delay in excretion is likely to represent the lag between absorption from the intestinal lumen and renal excretion. Although studies of

renal sucralose excretion have not yet been performed in humans, studies using lactulose have demonstrated a significant delay between intravenous administration and urinary excretion (Elia *et al*, 1987, Maxton *et al*, 1986).

This finding casts doubt upon the practice of using "delayed" probe excretion as a measure of colonic permeability. In a study investigating the effect of nicotine patches and NSAID's on the gastrointestinal tract, Suenaert *et al* reported 6-24 hour urinary sucralose excretion as being synonymous with colonic permeability (Suenaert *et al*, 2003). However, the results of the current study indicate that a significant proportion of 6-24 hour sucralose excretion may have originated from absorption in the small intestine, rather than the colon.

The 24-hour excretion of ^{51}Cr-EDTA in control subjects (median 2.73%) was higher than that in ileostomists (median 2.06%), by a factor of 1.32. The absolute difference (representing hypothetical colonic ^{51}Cr-EDTA absorption) was 0.669%. This implies that approximately a quarter of ^{51}Cr-EDTA permeation occurred in the colon of healthy control subjects.

Summary

The results of permeability tests in control subjects and ileostomists confirm sucralose and ^{51}Cr-EDTA to be markers of "whole-intestinal" permeability. It is estimated that a third of 24-hour sucralose excretion is the result of colonic absorption. Regrettably, the triple sugar test cannot be used to give an isolated measure of colonic permeability due to the confounding factor of colonic lactulose absorption. However, in the situation of a normal five-hour L/R ratio, an increase in 24-hour sucralose excretion is likely to be indicative of raised colonic permeability.

The results of testing ileostomists and control subjects also suggest that a significant amount of lactulose is absorbed in the colon. The results of previous studies involving lactulose/mannitol or lactulose/rhamnose tests may need to be re-interpreted in the light of this unexpected finding.

3.2. Other Patient Groups

Following the administration of the triple sugar test to control subjects and ileostomists, the test was administered to five groups of patients. Patients with a diagnosis of Crohn's disease, acute colitis and irritable bowel syndrome were selected as they had primary gastrointestinal pathologies which might be expected to result in alterations in intestinal permeability. Patients with acute pancreatitis and patients receiving adjuvant chemotherapy following surgery for breast cancer were selected as they suffered from disorders which might be expected to result in secondary gut injury. Evidence from the literature regarding permeability changes in these conditions is reviewed in section 1.5.4. ^{51}Cr-EDTA was used as an independent measure of "whole gut" permeability in subjects with Crohn's disease, acute colitis and irritable bowel syndrome. Medication usage by study participants is summarised in Table 9. Urine volumes are summarized in Table 10. Full permeability data for the five groups of patients are given in Table 11.

3.2.1. Crohn's Disease

3.2.1.1. Patients

Sixteen patients (5 women) with Crohn's disease were identified from a Gastroenterology outpatient clinic. Subjects were included on the basis of a prior endoscopic and histological diagnosis of Crohn's disease. Only subjects living at home who claimed to be in remission were considered for inclusion. Subjects who had previously undergone right hemicolectomy or small bowel resection six months or more prior to the study were included; subjects who had undergone more extensive or recent resection were excluded.

The median age of subjects with Crohn's disease was 51 years (IQR 44-65, p=0.064 *versus* control subjects, Mann-Whitney U). The median length of time between first diagnosis and administration of the triple sugar permeability test was 120 months (IQR 51-177). Seven patients had undergone previous right hemicolectomy, of whom four had undergone additional small bowel resections.

Disease severity was scored using a validated simple index of Crohn's disease activity (Harvey *et al*, 1980). The components of this score are shown in Table 7. The median score was three (IQR 1-6). This corresponds to a Crohn's Disease Activity Index of approximately 100, confirming that the majority of patients had quiescent disease (Harvey *et al*, 1980).

Table 7. Clinical scoring system for the simple index of Crohn's disease activity

(Harvey & Bradshaw 1980)

Symptom	*Score*
General Wellbeing	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Abdominal Pain	
None	0
Mile	1
Moderate	2
Severe	3
Abdominal Mass	
None	0
Dubious	1
Definite	2
Definite and Tender	3
Number of Liquid Stools/day	1 per stool
Extracolonic features	1 per manifestation

No subject had received non-steroidal anti-inflammatory medication within 48 hours of permeability testing, or alcohol within 24 hours. Medication usage is outlined in Table 9.

All subjects with Crohn's disease were offered a ^{51}Cr-EDTA test within two weeks of the triple sugar test. Three subjects declined, and a further subject failed to complete a 24-hour urine collection, leaving 12 subjects with ^{51}Cr-EDTA data.

3.2.1.2. Results

Lactulose and L-Rhamnose

The five-hour L/R excretion ratio was twofold greater in patients with Crohn's disease than in control subjects (0.0607 (0.0352-0.107) *versus* 0.0244 (0.0216-0.0350), p=0.007). This was due exclusively to an increase in lactulose excretion, as L-rhamnose excretion was similar in both groups (6.58% (3.78-8.50) in Crohn's group *versus* 6.98% (4.93-8.49) in controls, p=0.658). Twenty-four hour lactulose excretion and the 24-hour L/R ratio were similar between the two groups.

Sucralose

Twenty-four hour sucralose excretion was significantly higher in the Crohn's group than in controls (2.29% (1.59-2.84) *versus* 1.41% (1.12-1.68), p=0.001,). In contrast, five-hour sucralose excretion was similar in both groups. The proportion of sucralose excretion that occurred in the first five hours was significantly lower in subjects with Crohn's disease than in control subjects (39.0% (31.4-52.7) *versus* 53.0% (46.3-59.9), p=0.026).

^{51}Cr-EDTA

In keeping with sucralose excretion, 24-hour ^{51}Cr-EDTA excretion was significantly higher in subjects with Crohn's disease than in control subjects (4.12% (3.01-5.49) *versus* 2.73 (1.94-3.82), p=0.017).

3.2.1.3. Discussion

Subjects with Crohn's disease in remission were selected for study as they were readily accessible and could be seen on an outpatient basis. As discussed in section 1.5.2, permeability to both lactulose and ^{51}Cr-EDTA has been shown to be markedly raised in Crohn's disease. Results remained elevated when subjects were in clinical remission (Pironi *et al*, 1990, Wyatt *et al*, 1993). No attempt was made to relate permeability results to site of disease, for three reasons. Firstly, Crohn's is a whole gut disease, and so it is possible that permeability defects exist even in areas of the intestine which are not overtly inflamed. Secondly, subjects were in remission, and so the intestine did not contain identifiable areas of macro-inflammation. Thirdly, the sample size was too small to permit meaningful sub-group analyses.

Lactulose and L-Rhamnose

Five-hour lactulose and five-hour L/R ratio were both significantly increased in subjects with Crohn's disease, indicating a small-intestinal permeability defect. This is in keeping with the results of several previous studies (section 1.5.2). As patients were in remission, this increase in small intestinal permeability might have represented either sub-clinical inflammation or a baseline abnormality of the small intestine in subjects with Crohn's disease. Wyatt *et al* elegantly demonstrated that the lactulose/mannitol excretion ratio in Crohn's subjects in remission correlated with the likelihood of developing a relapse within one year, indicating that raised permeability in remission was probably an indicator of subclinical disease, rather than a baseline abnormality (Wyatt *et al*, 1993). In addition, theories of a genetically-determined permeability defect in subjects with

Crohn's disease and their first-degree relatives have largely been disproved (section 1.5.2.4).

In contrast, 24-hour lactulose excretion and the 24-hour L/R ratio in subjects with Crohn's disease were similar to those seen in control subjects. In the context of elevated five-hour lactulose excretion, this result implies that the colonic absorption of lactulose was less in subjects with Crohn's than in control subjects. Whilst it is possible that this was due to a reduction in colonic permeability to lactulose, this seems unlikely, given the fact that 24 hour permeability to sucralose and ^{51}Cr-EDTA was so markedly elevated. A more likely explanation for a reduction in colonic lactulose absorption is that there was a greater degree of colonic fermentation in subjects with Crohn's disease than in control subjects.

As colonic fermentation is difficult to quantify and subject to variation with changes in colonic microflora, the measurement of delayed lactulose excretion (after five hours) as a measure of intestinal permeability appears to be of questionable value. Further evidence of the confounding effect of bacterial fermentation on the measurement of colonic permeability to lactulose is discussed below.

Sucralose

Twenty-four hour sucralose excretion, a measure of whole-intestinal permeability, was significantly higher in subjects with Crohn's disease than in control subjects. This finding was in keeping with that of elevated ^{51}Cr-EDTA excretion (section 1.5.2.2), and was indicative of an increase in intestinal permeability over all, or part, of the gastrointestinal tract. Some of the increase in 24-hour sucralose excretion is likely to have originated from the small intestine, as small intestinal permeability to lactulose (five-hour L/R ratio, see above) was significantly increased. If this were the sole explanation, the increase in sucralose excretion would be expected to have occurred early in the 24-hour collection period. However, the proportion of 24-hour excretion which occurred in the first five hours of collection was only 39.0% (IQR 31.4-52.7), which was significantly lower than that seen in control subjects (53.0% (46.3-59.9), p=0.026). This finding suggests an increase in sucralose absorption towards the end of the collection period, in keeping with increased colonic permeability. Thus the increase in 24-hour sucralose excretion seen in subjects with Crohn's disease is likely to have been the result of an increase in both small intestinal and colonic permeability, as might be expected in this condition.

Interestingly, five-hour sucralose excretion was not elevated in subjects with Crohn's, in contrast to five-hour lactulose excretion or the five-hour L/R ratio. A similar dissociation between five-hour sucralose excretion and five-hour lactulose excretion was seen in ileostomists, who demonstrated a significant reduction in five-hour sucralose excretion *versus* controls (Figure 26) in the presence of a normal five-hour L/R excretion ratio

(Figure 24) and normal five-hour lactulose excretion (Figure 25). These data suggest that five-hour sucralose excretion is not a reliable indicator of small intestinal permeability, and that sucralose does not behave in an identical manner to lactulose during the first five hours of collection. There are many possible explanations for this. Much is unknown about sucralose, its site and method of absorption, and the effect of factors such as osmolality on absorption in the small intestine. Furthermore, there is little data regarding the pharmacokinetics of urinary excretion in humans. It may be that sucralose is absorbed more slowly than lactulose. Another possibility is that sucralose is only absorbed in the distal part of the small intestine, or that renal excretion is delayed. It is possible that sucralose is absorbed by a different route than lactulose, although this is considered unlikely, due to the chemical and structural similarities of the two molecules.

^{51}Cr-EDTA

In keeping with sucralose excretion, 24-hour ^{51}Cr-EDTA excretion was significantly higher in subjects with Crohn's disease than in control subjects. In the light of an elevated five-hour L/R excretion ratio, a significant part of the increase in ^{51}Cr-EDTA absorption is likely to have been the result of small intestinal absorption. The relative contribution made by colonic absorption cannot be estimated from the data available.

3.2.2. Acute Colitis

3.2.2.1. Patients

Eighteen subjects (10 women) admitted to hospital with acute colitis underwent the triple sugar test. The median age of these subjects was 52 years (IQR 45-59), which was significantly older than control subjects (p=0.032, Mann-Whitney U). All patients admitted with clinical features of acute colitis were considered for entry into the study. Further inclusion criteria were endoscopic and histological evidence of colitis in the absence of Crohn's disease or small intestinal pathology. Fourteen subjects had a final diagnosis of ulcerative colitis; five of these were new presentations. Two patients presented for the first time with ischaemic colitis, and a further two with distal colitis of indeterminate type.

Disease severity was scored using a validated simple colitis activity index (Walmsley *et al*, 1998). The components of this score are shown in Table 8. The median score was 11 points (IQR 8-13), indicating a high proportion of patients with severe disease. Four patients underwent acute colectomy, the remaining 14 responded to conservative treatment and were discharged home. There were no deaths.

Table 8. Clinical scoring system for the simple clinical colitis activity index

(Walmsley *et al*, 1998)

Symptom	*Score*
Bowel frequency (day)	
1-3	0
4-6	1
7-9	2
>9	3
Bowel frequency (night)	
1-3	1
4-6	2
Urgency of defaecation	
Hurry	1
Immediately	2
Incontinence	3
Blood in stool	
Trace	1
Occasionally frank	2
Usually frank	3
General well being	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extracolonic features	1 per manifestation

All triple sugar tests were performed within five days of admission to hospital. Twelve patients subsequently underwent the ^{51}Cr-EDTA test (performed within seven days of admission to hospital).

No subject had received non-steroidal anti-inflammatory medication (NSAID's) within 48 hours of permeability testing, or alcohol within 24 hours. Medication usage is outlined in Table 9.

3.2.2.2. Results

Lactulose and L-Rhamnose

Subjects with acute colitis demonstrated an increase in the five-hour L/R excretion ratio, however this did not reach statistical significance (p=0.071). In contrast, colitics demonstrated a significant reduction in the 24-hour L/R ratio (0.0369 (0.0254-0.0850) in colitics *versus* 0.0690 (0.0515-0.101) in controls, p=0.043), which was the result of reduced lactulose excretion.

Sucralose

Four out of the 18 colitics (22%) demonstrated 24-hour sucralose excretion greater than the 95th centile for control subjects. Despite this fact, the median 24-hour sucralose excretion in colitics was similar to that of control subjects (1.39% (1.08-2.52) *versus* 1.41% (1.12-1.68), p=0.735).

The proportion of 24-hour sucralose excretion which occurred during the first five hours of collection was significantly lower in colitics than in control subjects (41.1% (24.8-55.8) *versus* 53.0% (46.3-59.9), p=0.023).

^{51}Cr-EDTA

In keeping with 24-hour sucralose excretion, there was no significant difference in 24-hour ^{51}Cr-EDTA excretion between colitics and control subjects (p=0.181).

3.2.2.3. Discussion

Subjects with acute colitis were selected for study as they represented a population with site-specific intestinal disease which might be expected to result in increased colonic permeability (section 1.5.2.3). Subjects with Crohn's, or any other small intestinal pathology were excluded. Subjects with acute colitis requiring admission to hospital were chosen on the basis that they had the most severe disease, and were therefore likely to have more pronounced permeability changes than subjects who were managed as outpatients. As the triple sugar test was the main focus of this thesis, this was performed first (usually 2-5 days before the ^{51}Cr-EDTA test). It is recognized that subjects were likely to have improved clinically during the intervening few days.

Lactulose and L-Rhamnose

Five-hour lactulose excretion and five-hour L/R ratio were similar between subjects with acute colitis and control subjects, confirming the absence of small-intestinal disease. Twenty-four hour lactulose excretion and the L/R ratio were significantly reduced. This was not in keeping with sucralose or ^{51}Cr-EDTA excretion, and is likely to have been due to a confounding factor such as a change in bacterial fermentation or transit between colitics and controls. These data again confirm that delayed lactulose excretion is not a good marker of colonic permeability.

Sucralose

The presence of colonic inflammation is known to increase colonic permeability as measured by the rectal instillation of non-fermented probes such as ^{51}Cr-EDTA (section 1.5.2.3). If sucralose is truly a marker of whole gut permeability, then it would be expected that 24-hour excretion would be elevated in acute colitis. However, this anticipated increase in 24-hour sucralose excretion was not seen in the current study. The median 24-hour excretion in colitics was 1.39% (1.00-2.52), and in control subjects was 1.41% (1.12-1.68), p=0.735.

These data imply either that colonic permeability was not elevated in subjects with acute colitis, or that permeability was increased but the triple sugar test was not sensitive enough to detect it. The second of these possibilities is considered the most likely, for the following reasons. Four out of the 18 colitics (22%) demonstrated a 24-hour sucralose excretion greater than the 95th centile for control subjects (2.55%). The ^{51}Cr-

EDTA test was performed in two of these four subjects, and also found to be greater than the 95th centile for control subjects. Furthermore, the time-course of sucralose excretion was significantly altered in colitics as compared with controls, with a significantly lower proportion of 24-hour sucralose excretion occurring during the first five hours. This implies a shift from small-intestinal absorption to colonic absorption, which is suggestive of raised colonic permeability.

It can therefore be concluded subjects with acute colitis probably did have abnormal colonic permeability, but that the measurement of 24-hour sucralose excretion after an oral dose was not sensitive enough to detect this. There are several possible reasons for this. Ulcerative colitis spreads in a confluent manner from the rectum proximally as disease severity increases. Thus every colitic has left-sided disease, wheras only a few might have been expected to have right-sided disease. The left side of the colon is the most distal. By the time permeability probes have reached this area they will have been diluted and mixed with intestinal contents. Although the oral dose of sugar probes probably arrives in the small intestine as a bolus, the arrival of sugar probes in the left colon is likely to be far more gradual. This may have reduced probe absorption in the left colon, due to a reduction in trans-epithelial concentration gradients of probe sugars. One possible way to increase colonic sucralose concentration would be the use of a modified release preparation. The increasing number of peptide and protein drugs available for human use has resulted in the investigation of a number of colon-specific drug delivery systems, many of which currently exist only as prototypes. These preparations rely on a specific colonic "trigger" to effect release of an active drug, which either acts locally on

the colon in conditions such as colitis or colonic cancer, or is absorbed in a controlled-release manner into the systemic circulation to treat such conditions as asthma, angina and arthritis (Reddy *et al*, 1999). Triggering systems commonly employed include bacterial degradation (Liu *et al*, 2003), enzymatic cleavage of prodrugs (Sinha *et al*, 2003), and the use of a specific pH-degradable polymer coating (Reddy *et al*, 1999). Each triggering system has its drawbacks, and at the present time there is no colon-specific delivery system available for the administration of sugar probes.

Another factor with might have reduced the sensitivity of the triple sugar test in detecting raised colonic permeability is abnormal colonic transit. Studies in humans using radio-opaque markers have demonstrated that patients with acute ulcerative colitis have evidence of proximal colonic stasis, with rapid transit through the diseased segment (Allison *et al*, 1991, Rao *et al*, 1987, Reddy *et al*, 1991). This abnormal transit pattern might be expected to result in a reduction in the time sucralose is in contact with diseased colonic epithelium. This might in turn reduce sucralose absorption across diseased epithelium, counteracting the effect of increased epithelial permeability and resulting in normal (or even low) urinary sucralose excretion.

One way of overcoming the effect of variable colonic transit on 24-hour sucralose excretion might be to administer sucralose in combination with another probe molecule and measure fractional excretion, in a similar manner to the lactulose/rhamnose or lactulose/mannitol test. Any variation in transit time should affect the excretion of both probes equally, and so the excretion ratio would remain unchanged. In addition to the

properties of a permeability probe described in section 1.4.1.1, such a second "controlling" probe molecule would have to fulfill two main criteria. It would have to be absorbed via a different route to sucralose and would have to resist bacterial fermentation. The ideal "control" molecule would be a non-fermented monosaccharide, however no such probe has currently been identified. The only previously used permeability probe which is not a substrate for colonic fermentation and (arguably) is not absorbed by the paracellular route is PEG 400. PEG 400 has been used in combination with ^{51}Cr-EDTA to measure "whole gut" permeability in healthy volunteers (Peeters *et al*, 1994). The results were expressed as the ratio of 24-hour ^{51}Cr-EDTA/PEG 400 excretion The possibility of using a sucralose to PEG 400 excretion ratio merits investigation, allowing for the fact that the route of absorption of PEG polymers is uncertain (section 1.4.2.3).

^{51}Cr-EDTA

As mentioned above, the median 24-hour excretion of ^{51}Cr-EDTA in acute colitics was not significantly different to that seen in control subjects (1.80% (1.15-3.83)) *versus* 2.73% (1.94-3.82), p=0.181). Although several studies have demonstrated an increase in ^{51}Cr-EDTA excretion in subjects with ulcerative colitis (section 1.5.2.2), this has not been a universal finding (Bjarnason *et al*, 1983, Peled *et al*, 1985). Reasons for the failure to demonstrate a difference in permeability in subjects who had confirmed colonic inflammation in the current study are likely to be the same as for sucralose.

3.2.3. Irritable Bowel Syndrome

3.2.3.1. Patients

Eleven subjects (5 women) with irritable bowel syndrome, as defined by the Rome-two criteria, (Drossman *et al*, 1999) were identified from a gastroenterology outpatient clinic. The median (IQR) age was 60 years (54-68), which was significantly older than control subjects (p=0.003, Mann-Whitney U). All patients had been symptomatic for over a year. One subject had suffered a duodenal peptic ulcer some years prior to the study, other than this no subject had a past history of gastrointestinal disease.

No subject had a history of renal disease. No subject had received non-steroidal anti-inflammatory medication (NSAID's) within 48 hours of permeability testing, or alcohol within 24 hours. Medication usage is outlined in Table 9.

All subjects with irritable bowel syndrome were offered a ^{51}Cr-EDTA test within two weeks of the triple sugar test. One subject declined, leaving 10 subjects with ^{51}Cr-EDTA data.

3.2.3.2. Results

Lactulose and L-Rhamnose

The five-hour L/R excretion ratio in subjects with irritable bowel syndrome was similar to that seen in control subjects (0.0284 (0.0235-0.0543) *versus* 0.0244 (0.0216-0.0350) respectively, p=0.394). However, the 24-hour L/R ratio was significantly lower in subjects with irritable bowel syndrome (0.0446 (0.0372-0.0615) *versus* 0.0690 (0.0515-

0.101) in controls, p=0.037). This was due to a reduction in lactulose excretion over the latter part of the collection.

Sucralose

There were no significant differences between subjects with irritable bowel syndrome and control subjects in terms of five-hour sucralose excretion (p=0.439), 24-hour sucralose excretion (p=0.736), or the proportion of 24-hour sucralose excretion which occurred during the first five hours of collection (p=0.092).

^{51}Cr-EDTA

Twenty-four hour excretion of ^{51}Cr-EDTA was lower in subjects with irritable bowel syndrome than control subjects (1.92% (1.55-3.21) *versus* 2.73% (1.94-3.82)), however this was not statistically significant (p=0.090).

3.2.3.3. Discussion

Whilst many clinicians believe irritable bowel syndrome to be a purely psychosomatic condition, there is some evidence to suggest a pathophysiological cause (section 1.5.3). One proposed mechanism is that of mucosal micro-inflammation triggered by food antigens or infectious agents. As the symptoms of irritable bowel syndrome relate mainly to colonic function, the aim of using the triple sugar test in subjects with irritable bowel syndrome was to investigate any potential alteration in colonic permeability in this condition.

Lactulose and L-Rhamnose

There was no difference in five-hour L/R ratio between subjects with irritable bowel syndrome and healthy controls. This finding has been previously documented. In fact, dual sugar permeability tests have been used to differentiate between subjects with "organic" pathology and those with irritable bowel syndrome (section 1.5.3). In the current study 24-hour lactulose excretion was significantly lower in subjects with irritable bowel syndrome than in controls. This was the only abnormal permeability parameter, and is likely to have been due to confounding factors such as a difference in colonic lactulose fermentation between control subjects and those with irritable bowel syndrome.

Sucralose

Twenty-four hour sucralose excretion in subjects with irritable bowel syndrome was similar to that in control subjects. In the presence of normal small intestinal permeability (as demonstrated by a normal five-hour L/R ratio) this implies normal colonic

permeability. This is in keeping with a functional or psychosomatic basis for the symptoms of irritable bowel syndrome, rather than colonic micro-inflammation. However, a number of authors have demonstrated an increase in colonic mast cells and other inflammatory cells in subjects with irritable bowel syndrome (Gwee *et al*, 1999, Mayer *et al*, 2002, Spiller *et al*, 2000). It is possible that colonic micro-inflammation existed, but was not detectable using the triple sugar test.

^{51}Cr-EDTA

Twenty-four hour ^{51}Cr-EDTA excretion was not significantly different between subjects with irritable bowel syndrome and control subjects. Reasons for this are likely to be the same as for sucralose.

Summary

The only abnormal permeability parameter in subjects with irritable bowel syndrome was 24-hour lactulose excretion, which again confirms that the measurement of delayed lactulose excretion is unhelpful in the assessment of intestinal permeability. These data do not support the hypothesis that irritable bowel syndrome is the result of colonic micro-inflammation.

3.2.4. Acute Pancreatitis

3.2.4.1. Patients

Nine subjects (five women) who were admitted to hospital with a diagnosis of acute pancreatitis underwent the triple sugar test. The median (IQR) age of these subjects was 51 years (44-67) ($p=0.052$ compared to controls, Mann-Whitney U). Diagnosis was confirmed by a combination of suggestive history and serum amylase concentration of greater than four times the upper limit of normal (B.S.G., 1998). Disease severity was assessed during the first 48 hours following admission using the Glasgow scoring system (Blamey *et al*, 1984). Three or more positive criteria based on initial admission score and subsequent repeat tests constituted "severe" disease (B.S.G., 1998).

Using the Glasgow criteria one subject had severe disease. One further subject had evidence of severe pancreatic necrosis on subsequent CT scan. The remaining seven subjects were classified as having mild disease. The underlying cause was gallstones in six subjects and "idiopathic" in three subjects. One of the subjects with severe disease required treatment in an Intensive Care Unit (ICU). A further patient recovered from pancreatitis but suffered an anaphylactic reaction to anaesthetic agents during a laparoscopic cholecystectomy and required treatment in an ICU postoperatively. The remaining seven subjects made an uncomplicated recovery and were discharged home. There were no deaths.

The median length of time between admission to hospital and the triple sugar test was 3 days (IQR 1-6). No subject had received non-steroidal anti-inflammatory medication

(NSAIDs) within 48 hours of permeability testing, or alcohol within 24 hours. Medication usage is outlined in Table 9. C-Reactive Protein (CRP) was measured within 48 hours of admission in Scarborough Hospital Biochemistry Laboratory. C-Reactive Protein measurements were performed using an autoanalyser (COBAS Integra 400, Roche Diagnostics Ltd, Lewes, East Sussex). This machine underwent regular quality control, and performed with a coefficient of variation of 1.9-2.7%. The median CRP in subjects with acute pancreatitis at the time the triple sugar test was performed was 87mg/ml (IQR 53-106).

3.2.4.2. Results

Lactulose and L-Rhamnose

Subjects with acute pancreatitis demonstrated a marked increase in the five-hour L/R excretion ratio (0.0639 (0.0383-0.0911) *versus* (0.0244 (0.0216-0.0350), p=0.004). This was the result of both an increase in lactulose excretion and a reduction in L-rhamnose excretion. In contrast, the 24-hour L/R ratio was similar in subjects with pancreatitis and control subjects (0.0555 (0.0330-0.116) *versus* 0.0690 (0.0515-0.101), p=0.769).

Sucralose

Twenty-four hour sucralose excretion in subjects with acute pancreatitis was twice that of control subjects (2.80% (1.78-3.77) *versus* 1.41% (1.12-1.68), $p<0.001$). In contrast, five-hour sucralose excretion was similar in both groups ($p=0.839$). The proportion of 24-hour sucralose excretion which occurred during the first five hours of collection was significantly lower in pancreatitics than in control subjects (27.4% (17.3-60.0) *versus* 53.0% (46.3-59.9), $p=0.049$).

3.2.4.3. Discussion

Acute pancreatits accounts for 3% of all cases of abdominal pain admitted to hospital in the UK and represents a significant source of morbidity and mortality (B.S.G., 1998). Acute pancreatits provides a model for the study of systemic inflammation and gut-derived sepsis. In common with victims of burns and trauma, patients with acute pancreatitis exhibit a marked early inflammatory response, which can result in the development of the systemic inflammatory response syndrome (SIRS). Following adequate resuscitation, this "first-hit" is often followed by the development of single or multiple organ failure. Sepsis, in the form of infected pancreatic necrosis, is a serious later complication with a mortality rate of 40-70%. The causative organisms are usually gut-derived bacteria (Bassi *et al*, 2003, Garg *et al*, 2001, Isaji *et al*, 2003).

Acute pancreatitis, therefore, represents a model of systemic inflammation arising initially from a non-gastrointestinal source. In the "gut origin of sepsis" model, secondary gut injury and breakdown of the gut barrier are hypothesised to result in the

release of inflammatory mediators and colonic bacteria, perpetuating systemic inflammation and resulting in distant sepsis.

The purpose of including subjects with acute pancreatitis in the current study was to investigate the effect of pancreatitis on gut barrier function, with particular reference to colonic barrier function. Whilst subjects with severe disease might be expected to exhibit greater derangement of gut barrier function (Juvonen *et al*, 2000), mild disease is far more common. Subjects with both mild and severe disease were therefore included for study in order to obtain a sufficient sample size. The triple sugar test was performed a median of three days following admission; permeability data therefore relate to the early patho-physiological response. The presence of a significant degree of systemic inflammation at the time of testing was confirmed by a median CRP level of 87 mg/ml (normal reference range <8mg/ml).

Lactulose and L-Rhamnose

Subjects with acute pancreatitis demonstrated a marked increase in the five-hour L/R excretion ratio. This was in keeping with the results of several previous studies, which have documented an early and marked increase in small-intestinal permeability in pancreatitics (Ammori *et al*, 1999, Juvonen *et al*, 2000, McNaught *et al*, 2002). This increase in permeability is evidence of "secondary" gut injury. "Primary" gastrointestinal conditions which are consistently associated with a similar increase in small intestinal permeability include Crohn's disease and coeliac disease; both of which involve mucosal inflammation. It seems likely, therefore, that the increase in permeability seen in acute

pancreatitis is related to the effect of inflammatory mediators on the intestinal mucosa. Two potential routes for such mediators to reach the intestinal epithelium are contiguous spread from the inflamed pancreas, and release of inflammatory mediators from the pancreas into the systemic circulation, and thence to the gut. Whilst either or both of these routes may be clinically important, the second of these possibilities seems the more likely, as a similar increase in small intestinal permeability is seen in conditions which do not involve intra-abdominal inflammation, such as severe burns (section 1.5.4).

Sucralose

A marked early rise in 24-hour sucralose excretion was seen in pancreatitics. As in the case of subjects with Crohn's disease, this increase in "whole gut" permeability could have been the result of an increase in small intestinal permeability, colonic permeability, or a combination of the two. There was certainly evidence of increased small intestinal permeability, as measured using the five-hour L/R ratio. In addition to this, however, the proportion of 24-hour sucralose excretion which occurred during the first five hours of collection was significantly lower in pancreatitics than in control subjects. This finding, which was also demonstrated in subjects with Crohn's disease and acute colitis, implied an increase in sucralose absorption towards the end of the collection period, in keeping with increased colonic permeability. These data raise the interesting possibility that the colon might be a potential therapeutic target in patients with acute pancreatitis.

Colon-Specific Therapies in Acute Pancreatitis

One way of modulating the colonic barrier is through the use of probiotics. Probiotics are live microbial supplements which have a beneficial effect on the host by altering GI flora (Macfarlane *et al*, 2002). If an early increase in colonic permeability is indicative of colonic damage, which may later result in translocation of bacteria and endotoxin, then modulation of colonic microflora to a less pathogenic composition might reduce translocation and subsequent sepsis. In a recent randomised study involving 45 patients with acute pancreatitis, Olah *et al* demonstrated that the administration of a probiotic preparation containing *L. plantarum 299* was associated with a reduction in the incidence of infected pancreatic necrosis (Olah *et al*, 2002). This small study has to be set in the context of several other clinical trials, which have demonstrated no benefit from probiotic therapy (Anderson *et al*, 2004, McNaught *et al*, 2002).

One other potential gut-specific therapy is glutamine. Glutamine, a conditionally essential amino acid, is one of the major fuel sources for enterocytes (Hall *et al*, 1996, Windmueller *et al*, 1978). Glutamine has been shown to improve outcome in patients receiving intravenous nutrition (Griffiths *et al*, 1997, Houdijk *et al*, 1998). Glutamine appears to exert a beneficial effect on a number of organ systems, and its exact mechanisms of action remain unclear. There is, however, a mounting body of evidence to suggest that one of the main therapeutic actions of glutamine is a beneficial effect on the gut barrier. Several animal studies have demonstrated glutamine to be protective against TPN-induced gut atrophy (Chen *et al*, 1994, O'Dwyer *et al*, 1989, Platell *et al*, 1993). In addition, glutamine has been demonstrated to protect against bacterial

translocation across epithelial cell monolayers (Clark *et al*, 2003), and following abdominal radiation in rodents (Souba *et al*, 1990). One small study in postoperative patients receiving intravenous nutrition demonstrated that glutamine protected against duodenal mucosal atrophy (van der Hulst *et al*, 1993). Of note in this study was the fact that there was a significant increase in small intestinal permeability in subjects given normal parenteral nutrition, which was not seen in those administered nutrition supplemented with glutamine.

Glutamine therefore appears to represent a gut-specific therapy which could be applied to patients thought to be at risk of developing gut-derived sepsis. The apparent increase in colonic permeability in patients with acute pancreatitis in the current study warrants further investigation into the potential clinical benefits of administering glutamine in such patients.

3.2.5. Chemotherapy

3.2.5.1. Patients

Seven female subjects undergoing adjuvant chemotherapy following surgery for carcinoma of the breast underwent the triple sugar test. The chemotherapy agents employed were fluorouracil (600mg/m^2), epirubicin (60mg/m^2) and cyclophosphamide (600mg/m^2). Eight doses were administered in a 21 day cycle.

The median age of subjects undergoing chemotherapy was 57 years (IQR 50-65), which was significantly older than that of the control group (p=0.019, Mann-Whitney U). The median length of time between surgery and the test was 2.5 months (IQR 1.75-3.75). The median length of time between commencing chemotherapy and the test was 1.5 months (IQR 1.0-3.0). The median length of time between the most recent dose of chemotherapy and the test was 7.0 days (IQR 7.0-8.5).

No subject had received non-steroidal anti-inflammatory medication (NSAID's) within 48 hours of permeability testing, or alcohol within 24 hours. Medication usage is outlined in Table 9.

3.2.5.2. Results

Lactulose and L-Rhamnose

The five-hour L/R excretion ratio was similar in subjects undergoing chemotherapy and control subjects (p=0.853). However, the 24-hour L/R ratio was significantly lower in subjects undergoing chemotherapy than in control subjects (0.0231 (0.0009-0.0542) *versus* 0.0690 (0.0515-0.101), p=0.021). This was primarily due to a reduction in lactulose excretion over the latter part of the collection.

Sucralose

Five-hour urinary sucralose excretion was significantly reduced in subjects undergoing chemotherapy compared with control subjects (0.370% (0.330-0.600) *versus* 0.720% (0.570-0.956), p=0.003). Twenty-four hour sucralose excretion was similarly reduced (0.92% (0.83-1.21) *versus* 1.41% (1.12-1.68), p=0.012). The proportion of 24-hour sucralose excretion which occurred during the first five hours was also lower in subjects undergoing chemotherapy than in control subjects (42.0% (35.5-49.6) *versus* 53.0% (46.3-59.9), p=0.047).

3.2.5.3. Discussion

Subjects undergoing chemotherapy represent a further group at risk of secondary gut injury. Subjects undergoing chemotherapy are immunosuppressed, and therefore at risk of infection from a variety of sources. In addition, chemotherapy is known to have adverse effects on intestinal structure and function (section 1.5.5). This potential combination of immunosuppression and damage to the gastrointestinal mucosa could, hypothetically, result in the unopposed release of colonic bacteria and/or endotoxin into the circulation.

The group of subjects undergoing chemotherapy represented a controlled and relatively homogeneous population. Subjects had no evidence of gastrointestinal disease. They had undergone potentially curative resection, and so could be expected to have very little or no remaining tumour burden. The triple sugar test was performed a median of seven days post-chemotherapy, as this is the time when maximal GI disturbance has been shown to occur (section 1.5.5).

Lactulose and L-Rhamnose

Small intestinal permeability, as measured by the five-hour L/R ratio, was not significantly different from that of control subjects. This implies that there was not a significant degree of small intestinal inflammation one week following FEC chemotherapy. These data are in contrast to those seen in patients with metastatic breast cancer, as well as a variety of haematological and soft tissue tumours undergoing chemotherapy (section 1.5.5). One difference between the current study and previous

studies is that subjects in the current study had minimal (or no) tumour burden. Previously, subjects with advanced malignancy have been investigated. It is possible that the presence of malignancy predisposes to an increase in permeability following chemotherapy. As it would be unethical to measure the effect of chemotherapy on intestinal permeability in healthy volunteers, direct evidence in support of this hypothesis is lacking. However, Sundstrom *et al* demonstrated an increase in small intestinal permeability in patients with acute myeloid leukaemia *before* treatment with chemotherapeutic agents (Sundstrom *et al*, 1998). Similarly, Parrilli *et al* demonstrated abnormal lactulose/rhamnose absorption in 10 patients with metastatic breast cancer before and after treatment with Adriamycin (Parrilli *et al*, 1989). It is possible that the presence of an intestinal permeability defect prior to the induction of chemotherapy might enhance epithelial damage, and hence result in a measurable increase in lactulose excretion.

Sucralose

Sucralose excretion was significantly reduced over both five and 24 hours in subjects undergoing chemotherapy compared with control subjects. This implied an abnormality of transit, absorption or excretion. These data were not in keeping with mucosal inflammation, which would be expected to result in an increase in sucralose excretion, similar to that seen in subjects with Crohn's.

As no subject had evidence of renal impairment, a primary abnormality of sucralose excretion was unlikely. One possible explanation for the reduction in urinary sucralose

was a reduction in intestinal transit time associated with a chemotherapy-induced increase in intestinal motility (Ippoliti, 1998). Increased transit would result in less contact time between sucralose and the intestinal epithelium, leading to less sucralose absorption. A further possibility is that of reduced epithelial surface area secondary to villus atrophy. Villous atrophy has been shown to occur as part of chemotherapy-induced mucositis (Keefe *et al*, 2000). Theoretically, such a reduction in surface area could account for reduced sucralose excretion. However, villous atrophy would have resulted in a reduction in L-rhamnose excretion, as seen in coeliac disease (section 1.5.1). This was not seen in subjects undergoing chemotherapy in the present study. Furthermore, villous atrophy is usually associated with an increase in immature tight junctions, which might be expected to increase sucralose absorption (Keefe *et al*, 2000).

Table 9. Medication usage by study participants

Group	N	Nil	NSAID's	Diuretics	Amino-salicylates	Gluco-corticoids	Immuno-suppressants
Control	21	20	0	0	0	0	0
Ileostomy	18	6	1	3	1	1	0
Crohn's	16	3	0	1	8	4	4
Colitis	18	1	2	2	12	12	2
Pancreatitis	9	1	2	1	0	1	0
IBS	11	3	4	1	0	0	0
Chemo	7	3	1	1	0	1	0

(Numbers represent number of subjects)

Table 10. Triple-sugar test urine volumes in all study participants

Group	Five-hour Volume (ml)	24-hour Volume (ml)
Control	**452** (212-715)	**1984** (1335-2539)
Ileostomy	**190** (105-352) *P=0.005*	**997** (662-1613) *P=0.007*
Crohn's	**380** (230-580) *P=0.432*	**1604** (1217-1950) *P=0.122*
Colitis	**450** (204-891) *P=0.883*	**2336** (1444-3246) *P=0.185*
IBS	**582** (222-886) *P=0.538*	**1718** (966-2342) *P=0.552*
Pancreatitis	**244** (125-437) *P=0.049*	**1544** (1225-3588) *P=0.803*
Chemotherapy	**196** (100-482) *P=0.106*	**1192** (510-2422) *P=0.194*

Numbers represent median (IQR). P-values refer to Mann Whitney-U test *versus* control subjects.

Table 11. Permeability results in all study participants

Urinary Excretion Rates Following Oral Administration	Control	Ileostomy	Crohn's	Colitis	IBS	Pancreatitis	Chemo
Five-hour Sucralose (%)	**0.720** (0.570-0.950)	**0.465** (0.340-0.743)	**0.814** (0.479-1.24)	**0.670** (0.385-0.817)	**0.570** (0.460-0.900)	**0.698** (0.415-1.755)	**0.370** (0.330-0.600)
P	N/A	0.013	0.665	0.252	0.439	0.839	0.003
24-hour Sucralose (%)	**1.41** (1.12-1.68)	**0.955** (0.630-1.24)	**2.29** (1.59-2.84)	**1.39** (1.08-2.52)	**1.37** (1.04-2.40)	**2.80** (1.78-3.77)	**0.92** (0.83-1.21)
P	N/A	0.003	0.001	0.735	0.736	<0.001	0.012
19-hr Sucralose (%)	**0.644** (0.503-0.925)	**0.405** (0.275-0.655)	**1.29** (0.911-1.52)	**0.670** (0.545-2.17)	**0.890** (0.340-1.43)	**1.32** (0.967-3.00)	**0.600** (0.397-0.642)
P	N/A	0.010	0.001	0.284	0.439	0.005	0.194
Five-hour Sucralose as % of 24-hour excretion	**53.0** (46.3-59.9)	**57.5** (34.4-67.7)	**39.0** (31.4-52.7)	**41.1** (24.8-55.8)	**41.6** (35.0-54.8)	**27.4** (17.3-60.0)	**42.0** (35.5-49.6)
P	N/A	0.517	0.026	0.023	0.092	0.049	0.047
24-hour Lactulose (%)	**0.517** (0.388-0.617)	**0.263** (0.159-0.388)	**0.445** (0.188-1.39)	**0.221** (0.192-0.660)	**0.462** (0.158-0.619)	**0.550** (0.240-1.16)	**0.270** (0.120-0.330)
P	N/A	0.003	0.939	0.026	0.293	0.946	0.002
24-hour Rhamnose (%)	**7.41** (5.47-10.32)	**8.22** (6.44-10.62)	**8.11** (6.21-10.75)	**7.34** (5.90-8.63)	**8.07** (6.19-11.36)	**6.90** (5.36-14.5)	**6.74** (6.09-11.67)
P	N/A	0.464	0.520	0.800	0.648	0.572	0.770
24-hour L/R Ratio	**0.0690** (0.0515-0.101)	**0.0311** (0.0179-0.0617)	**0.0549** (0.0293-0.188)	**0.0369** (0.0254-0.0850)	**0.0446** (0.0372-0.0615)	**0.0555** (0.0330-0.116)	**0.0231** (0.00930-0.0542)
P	N/A	0.004	0.806	0.043	0.037	0.769	0.021

(Continued)

Urinary Excretion Rates Following Oral Administration	Control	Ileostomy	Crohn's	Colitis	IBS	Pancreatitis	Chemo
24 hour ^{51}Cr-EDTA (%)	**2.73** (1.94-3.82)	**2.06** (1.53-2.75)	**4.12** (3.01-5.49)	**1.80** (1.15-3.83)	**1.92** (1.55-3.21)	N/A	N/A
P	N/A	0.037	0.017	0.181	0.090	N/A	N/A
Five-hour Lactulose (%)	**0.170** (0.125-0.270)	**0.149** (0.0845-0.192)	**0.370** (0.136-0.842)	**0.208** (0.121-0.285)	**0.156** (0.143-0.414)	**0.280** (0.104-0.385)	**0.140** (0.100-0.182)
P	N/A	0.210	0.092	0.662	0.796	0.511	0.212
Five-hour Rhamnose (%)	**6.98** (4.93-8.49)	**5.10** (3.36-7.13)	**6.58** (3.78-8.50)	**4.76** (3.18-7.04)	**7.07** (5.03-8.48)	**3.95** (2.81-6.11)	**4.85** (3.32-7.48)
P	N/A	0.047	0.658	0.041	0.890	0.007	0.185
Five-hour L/R Ratio	**0.0244** (0.0216-0.0350)	**0.0246** (0.0215-0.0371)	**0.0607** (0.0352-0.107)	**0.0448** (0.0234-0.0690)	**0.0284** (0.0235-0.0543)	**0.0639** (0.0383-0.0911)	**0.0275** (0.0190-0.0421)
P	N/A	0.955	0.007	0.071	0.394	0.004	0.853
Five-hour Lactulose as % of 24-hour excretion	**40.2** (24.2-51.2)	**60.1** (26.0-76.4)	**70.1** (45.1-99.7)	**77.2** (41.8-110.2)	**67.1** (37.1-100.0)	**64.3** (24.3-81.9)	**56.9** (42.4-166.7)
P	N/A	0.226	0.020	0.024	0.045	0.402	0.053
Five-hour Rhamnose as % of 24-hour excretion	**86.2** (80.0-96.6)	**66.7** (53.0-75.6)	**79.2** (65.4-89.6)	**76.3** (47.6-91.0)	**89.8** (59.6-97.1)	**46.5** (35.3-96.6)	**74.3** (46.4-118.0)
P	N/A	<0.001	0.107	0.121	0.766	0.018	0.194

Values represent median (IQR). P-values represent Mann-Whitney U test *versus* control subjects. P-values ≤0.05 are shown in red.
N/A = Not applicable
L/R = Lactulose/rhamnose

Part 4: Concluding Remarks

4.1. Main Conclusions

The aim of this study was to investigate the use of a triple sugar test of intestinal permeability as a surrogate marker of gut barrier function in surgical patients. It was hypothesized that the use of a non-fermented sugar (sucralose), in combination with a fermented sugar (lactulose) would allow quantification of small and large intestinal permeability separately. Experimental work included development of suitable laboratory methods for quantifying urinary test probes, administration of the triple sugar test to control subjects and ileostomists, and preliminary investigation of intestinal permeability in a number of patient groups. The results of the various experiments are discussed separately in detail above. The broader conclusions of the author are summarised below.

Conclusion 1: The triple sugar test enabled the simultaneous measurement of "whole gut" and small intestinal paracellular permeability.

The results of this work have identified two useful components of the triple sugar test; the five-hour L/R excretion ratio and 24-hour sucralose excretion. The study of controls and ileostomists demonstrated for the first time in humans that 24-hour sucralose excretion represents "whole gut" permeability, and confirmed that the five-hour L/R ratio represents small intestinal permeability. Twenty-four hour sucralose excretion behaved in an identical manner across the study groups to 24-hour ^{51}Cr-EDTA excretion, which

strongly suggests that sucralose, like ^{51}Cr-EDTA, is a marker of paracellular permeability.

Conclusion 2: **A significant amount of lactulose was absorbed in the colon in human subjects**

This study demonstrated for the first time that an appreciable amount of lactulose was absorbed in the colon in human subjects. This precluded the measurement of colonic permeability by simply subtracting 24-hour lactulose excretion from 24-hour sucralose excretion. Colonic lactulose absorption was unpredictable, presumably due to variation in colonic lactulose fermentation. This prevented meaningful interpretation of delayed urinary lactulose excretion (i.e. urinary excretion greater than five hours following oral ingestion). For this reason delayed lactulose excretion should not, in the author's opinion, be used as a measure of intestinal permeability.

Conclusion 3: **The triple sugar test was relatively insensitive at detecting isolated colonic damage.**

One drawback of the triple sugar test in the current study was the failure to demonstrate with certainty an increase in "whole gut" permeability in subjects with acute colitis, although alterations in the temporal excretion of sucralose were suggestive of increased colonic permeability. Potential methods of increasing sensitivity include the use of a non-fermented "control" probe and the administration of sucralose in a colon-specific

formulation. It is the recommendation of the author that these methods be investigated in order to increase sensitivity of the triple sugar test for colonic damage, prior to its use as a measure of colonic permeability in future work.

Conclusion 4: Acute pancreatitis was associated with increased colonic permeability

Despite the limitations of the triple sugar test, data from the current study suggested that acute pancreatitis was associated with an increase in colonic permeability. This important finding provides a rational for the use of colon-specific therapies in pancreatitis. Possible therapeutic strategies include the use of pre- and probiotics and the administration of gut-specific nutrients such as glutamine.

4.2. Additional Comments

This study has demonstrated that the triple sugar test is easy to perform in a wide variety of patient groups. The sites of probe absorption have been clearly defined, along with the limitations of the technique. Further advantages of the triple sugar test are that it is non-invasive, non-radioactive, and that repeated measures are possible in the same patient. As regards the clinical relevance of intestinal permeability, it has been repeatedly demonstrated that conditions which result in systemic inflammation, sepsis with enteric bacteria and multiple organ failure are associated with an early increase in intestinal permeability (section 1.5.4). The degree of increased permeability has been shown to correlate to disease severity and endotoxin exposure (Ammori *et al*, 1999, Juvonen *et al*, 2000, Ryan *et al*, 1992). This provides strong circumstantial evidence that an increase in intestinal permeability is a clinically relevant phenomenon, and may represent an important pathogenic step in breakdown of the gut barrier.

It is clear, however, that gut-derived sepsis cannot be accounted for by altered permeability alone. Whilst there is a definite correlation between increased permeability and systemic inflammation, a link between permeability and bacterial translocation has not been demonstrated in humans. A pre-operative increase in permeability has been shown to have no predictive value for either bacterial transloction or sepsis in patients undergoing abdominal surgery (Kanwar *et al*, 2000, O'Boyle *et al*, 1998). A study of patients with severe blunt trauma led to the authors to conclude that "...increased permeability and subsequent infectious complications are independent phenomena,

frequently seen in patients after severe trauma or hemorrhagic shock." (Roumen *et al*, 1993). Furthermore, there is an increasing body of evidence to suggest that bacterial translocation occurs in a trans- rather than para-cellular manner (Bras *et al*, 1999, Clark *et al*, 2003, Wells *et al*, 1995, Wells *et al*, 1996). Practical disadvantages to using the triple sugar test were that time-consuming analytical techniques were required for the quantification of urinary sugars, and that a direct measure of colonic permeability eluded the author.

Not withstanding these drawbacks, the author believes that the triple sugar test could become a useful research tool for investigating the gut barrier in humans. Serial measurements will allow assessment of the effect of gut-directed therapies such as pre- and probiotics or gut-specific nutrients on both the small and large intestine in clinical trials. Further refinement of clinical and laboratory techniques is likely to result in a test which has greater sensitivity for colonic damage and is relatively simple to perform. It is hoped that this contribution will increase understanding of intestinal permeability and will facilitate the use of non-fermented probes such as sucralose in combination with traditional lactulose/monosaccharide tests in future studies.

Acknowledgements

The author wishes to thank the following individuals, without whose help this thesis could not have been completed:

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Appendices

Appendix 1: Written Information Sheet for Study Participants

(Text Originally 13 Point)

Scarborough and North East Yorkshire Healthcare NHS
NHS Trust

Scarborough Hospital
Woodlands Drive
Scarborough
North Yorkshire
YO12 6QL

Tel: 01723 368111

PATIENT INFORMATION SHEET (non-surgical patients)

Measurement of Colonic Permeability

You are being invited to take part in a research study. Before you decide, it is important that you understand why the research is being done and what it will involve. Please read the following information and feel free to ask us anything else you would like to know.

Every person has millions of bacteria in their large bowel (colon). Normally, they don't do us any harm. However, current thinking is that some infections (like pneumonia, urinary infections and septicaemia) might be due to these bacteria escaping from the colon and travelling to other parts of the body. In order for this to happen, bacteria have to pass through small gaps in the bowel wall – in other words the colon has to be "leaky."

The purpose of this study is to measure this "leakiness" (permeability) of the colon. We are studying people with previous bowel disorders. We are also studying some normal people in order to compare results.

If you agree to be involved in the study we will ask you to take omit any food or drink after midnight. Before breakfast the next day we will give you a drink containing 2 different sugars and a tiny amount of radioactive material. Another sugar is given in capsule form. You will be able to eat and drink normally 2 hrs after taking this test drink. All the urine you pass in the next 24 hours will be collected, and we will measure how much of the sugars and radioactive material has passed from the bowel into the urine. The amount of radioactivity is not dangerous – it is less than a normal x-ray.

If you agree to be involved in the study, you will be asked to sign a consent form. Any data collected will be entirely confidential. If at any time you wish to withdraw from the study you may do so without giving a reason and it will not affect your future healthcare in any way. If you have any questions, or would like

to speak to a member of the research team, please feel free to contact Dr Sandy Anderson (phone 07811125082) at any time. Alternatively, feel free to discuss any questions you have with a member of the team looking after you in hospital.

You may keep this information sheet and you will be given a copy of the consent form.

Thank you for taking part in this study,

Sandy Anderson
Surgical Research Fellow
Scarborough Hospital

Appendix 2: Consent Form for Study Participants

(Text Originally 13 Point)

Scarborough and North East Yorkshire Healthcare NHS
NHS Trust

Scarborough Hospital
Woodlands Drive
Scarborough
North Yorkshire
YO12 6QL

Tel: 01723 368111

PATIENT CONSENT FORM

Measurement of Colonic Permeability

I confirm that I have read and understood the written information sheet regarding the above clinical study. The aims and objectives of the study have been explained to me.

I have received verbal information about the study and I have had an opportunity to discuss the study and ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

I agree to take part in the above study.

Patient's name _____

Signature _____

Date _____

I confirm that I have given the above patient information about this study.

Researcher _____

Signature _____

Date _____

Appendix 3: ARSAC License for the Administration of ^{51}Cr-EDTA

CERTIFICATE

FOR THE

ADMINISTRATION OF RADIOACTIVE MEDICINAL PRODUCTS

Certificate Reference Number RPC 382-2168 (16573)

It is hereby certified for the purposes of the Medicines (Administration of Radioactive Substances) Regulations 1978, amended by the Medicines (Administration of Radioactive Substances) Amendment Regulations 1995, that

Ian G H RENWICK
Scarborough Hospital
Woodlands Drive
Scarborough
North Yorkshire
YO12 6QL

may administer until 19 Mar 2004 the radioactive medicinal products specified in the Schedule to this certificate for the purpose(s) there specified.

for The Secretary of State for Health

....

Health Care Directorate
Specialist Clinical Services Division
Department of Health

20-Mar-2002

Ian G H RENWICK
Scarborough Hospital
Woodlands Drive
Scarborough
North Yorkshire
YO12 6QL

Date of
Certificate 20.03.02

Schedule to Research Certificate Number RPC 382-2168 (16573)

Research Project

An Investigation Into The Measurement of Colonic Permeability and its Association with Bacterial Translocation, Gut Immune Function and Systemic Inflammation.

Serial*	Nuclide	Chemical Form
24a 4i	51Cr	EDTA

*As listed in Appendix I of the Notes for Guidance.
Unlisted Serial numbers are included for ease of identification by the ARSAC Secretariat.

Appendix 4: Published Papers and Abstracts

Poster Presentation at the Society of Academic and Research Surgery 2004 (Belfast)

Abstract Published in SARS Yearbook 2004 p70

SARS

Abstracts

74 DEFICIENT INITIATION OF THE ACQUIRED IMMUNE RESPONSE AND NEUTROPHIL ACTIVATION IN PATIENTS WITH OBSTRUCTIVE JAUNDICE

R Bullock*, C Fiuza, G Aithal, A Robins, BJ Rowlands
Section of Surgery. Queen's Medical Centre. University Hospital. Nottingham

Background:
Patients with obstructive jaundice are at a high risk of sepsis and organ dysfunction after surgery. Understanding factors that initiate and modulate immune function may be useful in developing therapies for these patients.

Methods:
5 patients with obstructive jaundice and 6 healthy controls were included in the study. The expression of CD11b and L-selectin (markers of leukocyte activation) and HLA-DR (marker of monocyte antigen-presenting capacity, initial step of the acquired immune response) was quantified in whole blood by flow cytometry. A whole blood phagocytosis assay using FICT labelled *E. coli* was used to determine monocyte and neutrophil phagocytosis. The research protocol was approved by the local ethics committee. Results are expressed as mean ± standard deviation.

Results:
Compared to healthy controls, neutrophils from jaundiced patients had increased expression of CD11b (44,856± 21,239 vs 21,063±7,573 receptors/cell, p=0.02) and decreased expression of L-selectin (17,656±10,237 vs 37095±13,753 receptors/ cells, p = 0.02), suggesting neutrophil intravascular activation. Monocytes from jaundiced patients demonstrated increased levels of CD11b and a profound suppression of HLA-DR expression (3.451±2.436 vs 23.282±6.565 receptors/cell, p=0.01). There was no significant difference in *E. coli* neutrophil phagocytosis between the two groups, but monocyte phagocytosis was severely depressed in jaundiced patients (44.9% ±7.82 vs 70.65% ± 7.3 %, p=0.04).

Conclusions:
Patients with obstructive jaundice present signs of neutrophil intravascular activation with conserved phagocytosis while monocyte antigen presenting capacity and bacterial phagocytosis are impaired. A deficient initiation of the acquired immune response in the presence of neutrophil activation may contribute to the immune dysfunction in obstructive jaundice.

75 A TRIPLE SUGAR TEST OF INTESTINAL PERMEABILITY IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

ADG Anderson *, SC Fleming, PK Jain, J MacFie
Combined Gastroenterology Research Group, Scarborough Hospital, England

Background:
Established dual sugar tests of intestinal permeability (eg lactulose/ rhamnose) assess only small intestinal barrier function. We have used a novel triple sugar technique employing a combination of sucralose, lactulose and rhamnose to evaluate changes in both small intestinal and colonic permeability in patients with inflammatory bowel disease.

Methods:
Healthy controls (n=21), subjects with an acute exacerbation of ulcerative colitis (UC, n=14) and patients with Crohn's disease in remission (n=10) were administered the "triple sugar test." After an overnight fast subjects consumed a test drink containing sucralose (5g), lactulose (5g) and rhamnose (1g). Urine was collected for 24 hours in 2 aliquots (first 5 and last 19 hours). Urinary sugar concentrations were assayed by HPLC. All results are expressed as median (IQR), and differences between groups compared with the Mann-Whitney U test.

Results:
There were no significant differences in lactulose excretion or L/R ratio between the groups. There was a significant increase in 24hr sucralose excretion in patients with Crohn's disease (2.49% (1.98-3.37)) compared to control subjects (1.41% (1.12-1.68)) (p=0.001). Sucralose excretion was not elevated in patients with acute UC (1.39% (1.06-4.51)) (p=0.711 compared to controls). The proportion of sucralose excreted in the last 19hrs of collection was significantly greater in both groups compared to controls (p=0.047 Crohn's, p=0.049 UC).

Conclusions:
Twenty-four hour sucralose excretion was a more sensitive indicator of intestinal disease than conventional lactulose/rhamnose tests. There is a suggestion that the triple sugar test may enable detection of isolated colonic disease, through examination of temporal excretion rates.

Poster Presentation at the Association of Surgeons of Great Britain and Ireland 2003 (Manchester)

Abstract Published in BJS 2003; 90 (Supplement 1): 142

patients who underwent cardiac surgery from November 2000 to January 2001.

Methods: Patients were screened daily for evidence of culture positive wound, respiratory, urinary tract or other infections. Wounds were examined daily and defined on ASEPSIS score. Chest X-rays, white cell counts and differentials were recorded on days 1, 2, and 4. The use of blood was monitored blind and independently. Patients were grouped according to transfusion and compared using χ^2 or Fisher's test.

Results: Of 232 patients, 116 (50%) received allogenic blood transfusion. Patients who received blood were older had a greater portion of urgent/emergency or revision surgery and were higher risk. Despite this, there were no differences in the frequency of any postoperative infection. Forty-five patients received larger volumes ($\geq$ 4 units) of blood. There was again no difference in the frequency of infection.

	No transfusion	Transfusion	*P* value
Mean age in years, (s.d.)	62 (11)	66 (12)	0·01
Urgent/emergency procedure, *n* (%)	9 (7·8)	19 (16·4)	0·04
Median euroscore (i.q.r.)	3 (2 to 5)	5 (3 to 7)	< 0·001
Resternotomy, *n* (%)	4 (3·4)	13 (11·2)	0·02
Chest infection, *n* (%)	17 (14·8)	23 (19·8)	0·38
Wound infection, *n* (%)	9 (8·0)	4 (3·5)	0·16
Urinary tract infection, *n* (%)	6 (5·3)	4 (3·5)	0·75
Other infections, *n* (%)	8 (7·0)	8 (7·2)	1·0

Conclusions: In patients undergoing cardiac surgery, blood transfusion does not increase the risk of postoperative infection.

Poster 92

A triple sugar test of colonic permeability in surgical patients

A. D. G. Anderson, S. C. Fleming, P. K. Jain, C. J. Mitchell and J. MacFie

Combined Gastroenterology Research Group, Scarborough Hospital, Scarborough, UK

Aims: The aim of this study was to evaluate the use of a novel triple sugar technique for the measurement of colonic permeability. This technique is based on the principle that sucralose (a synthetic sugar) resists degradation by colonic bacteria whereas lactulose is stable only in the small intestine.

Methods: Twenty healthy volunteers and 16 subjects with established ileostomies were studied. After an overnight fast subjects ingested a test solution containing 5 g sucralose, 5 g lactulose and 1 g rhamnose. Urine was collected for 24 hours, and sugar concentrations assayed by HPLC. An independent measure of whole-intestinal permeability was obtained using ^{51}Cr-EDTA. Results are presented as the median percentage of probe excreted in the urine over 24hrs. Differences between groups were analysed using the Mann–Whitney *U* test.

Results: Median sucralose excretion in patients with ileostomies was half that of controls (median 0·77% *vs.* 1·41%, $P < 0.05$). Similar results were seen with ^{51}Cr-EDTA (1·90% excretion in ileostomists *vs.* 2·73% in controls, $P < 0.05$). Rhamnose excretion was the same in both groups but lactulose excretion was reduced in ileostomists, giving a median lactulose/ rhamnose ratio of 0·032 in ileostomists *vs.* 0·070 in controls ($P < 0.05$).

Conclusions: The triple sugar test described is a promising non-invasive way of assessing the relative contributions of small and large intestinal permeability to gut barrier function. The reduced lactulose/rhamnose ratio seen in patients with ileostomies may be the result of bacterial colonisation of the small bowel with subsequent fermentation of lactulose, rather than 'true' altered permeability.

Poster 93

Operative objectives of basic surgical trainees in general surgery

A. M. Paisley, P. J. Baldwin and S. Paterson-Brown

Department of Clinical and Surgical Sciences (Surgery), Royal Infirmary of Edinburgh, Edinburgh, UK

Aims: In previous work we have defined the operative skills expected of basic surgical trainees (BST) by consultant general surgeons. This study determines whether consultant expectation of BST experience is being met.

Methods: Over an 18-month period all general surgical SHOs on the SE Scotland basic surgical training scheme were asked to submit logbook data. These data were analysed to determine the total number of procedures in 6 months at which trainees were first assistant and those which they performed supervised and unsupervised. Level of experience in 7 key procedures, previously identified by consultant surgeons as those which SHOs 'must be able to perform unsupervised (4) and supervised (3)', was also determined.

Results: 34/40 (85%) logbooks were returned. In six months SHOs were involved in a median of 144 (range 121–170) procedures. Of these 31 (19–48) were performed unsupervised, 45 (39–52) were performed supervised and

Oral Presentation at the Association of Surgeons of Great Britain and Ireland 2004 (Harrogate)
Abstract Published in BJS 2004; 91 (Supplement 1): 44

principal diagnosis. The negative appendectomies were confirmed following pathology reports.

Results: A total of 10,142 appendectomies were recorded between June 1999 and December 2002. During this time period 2964 (29·2%) were classified as negative appendectomies. The NAR was higher in females (60%) than males (40%) with the highest incidence occurring between the ages of 11 and 20 years. The NARs of each hospital was in the range 15–89%.

Conclusions: In summary, it is clear that the NAR is highly variable throughout Wales. In the minority of hospitals the NAR is < 20%, but for most hospitals this is not the case. It is worrying that in four Welsh hospitals ≥ 50% of appendix cases are negative. It seems that great variability in practice patterns and resource utilisation exists in the management of acute appendicitis.

General 16

Gut barrier function: the simultaneous measurement of colonic and small intestinal permeability in surgical patients

A. D. G. Anderson, S. C. Fleming, P. K. Jain and J. MacFie

Combined Gastroenterology Research Group, Scarborough Hospital, Scarborough, UK

Aims: Intestinal permeability (IP) is often used as a surrogate marker of gut barrier function. Conventional methods of measuring IP employ dual sugar probes, but these undergo colonic fermentation and only permit assessment of small intestinal permeability. Using sucralose (a non-fermented sugar) we have developed a novel triple sugar test, which measures both colonic and small intestinal permeability.

Methods: Following an overnight fast, subjects consumed a test drink containing sucralose (5 g), lactulose (5 g) and rhamnose (1 g). Urine was collected for 24 hours in two aliquots (first 5 and last 19 hours). Urinary sugar concentrations were assayed by HPLC. The groups studied were: healthy volunteers ($n = 21$), patients with an ileostomy ($n = 18$), Crohn's disease ($n = 16$), acute colitis ($n = 18$), IBS ($n = 11$), acute pancreatitis ($n = 9$) and patients undergoing chemotherapy ($n = 7$).

Results: Small intestinal permeability (5-hour lactulose/rhamnose ratio) was increased in subjects with Crohn's disease ($P = 0·007$) and acute pancreatitis ($P = 0·004$), *versus* controls. 'Whole gut' permeability (24-hour urinary sucralose excretion) was significantly increased in patients with Crohn's ($P = 0·001$) and pancreatitis ($P < 0·001$), and significantly reduced in ileostomists ($P = 0·003$) and patients undergoing chemotherapy ($P = 0·012$). The proportion of sucralose excreted in the last 19 hours of collection was significantly increased in patients with Crohn's ($P = 0·026$), acute colitis ($P = 0·023$) and acute pancreatitis ($P = 0·049$), implying an increase in colonic permeability.

Conclusions: The triple sugar test allows simultaneous quantification of small intestinal and 'whole-gut' permeability. By examining temporal probe excretion rates, isolated colonic damage can be identified. This is the first description of a non-invasive method of measuring colonic permeability.

General 17

Fate of the inguinal hernia following removal of infected prosthetic mesh

A. S. Fawole, R. P. C. Chaparala and N. S. Ambrose

Department of Academic Surgery, St James's University Hospital, Leeds, UK

Aims: Open tension-free hernioplasty using a prosthetic mesh is a common operation for inguinal hernia repair because of the relative ease of the operation and low recurrence rate. Wound infection is a complication of all hernia repairs and deep-seated infection involving an inserted mesh may result in chronic groin sepsis, which usually necessitates complete removal of mesh to produce resolution. Removal of mesh would potentially result in a weakness of the repair and subsequent hernia recurrence. We reviewed the outcome of all the patients who had mesh removal for sepsis over an 8-year period in our hospital, particularly examining for hernia recurrence and chronic groin pain.

Methods: There were 2139 inguinal hernias repaired using a prosthetic mesh over an 8-year period. All patients who had mesh removal for infection were reviewed and followed up.

Results: Fourteen patients had deep-seated wound infection that required mesh removal for resolution of sepsis. No perioperative complications occurred during mesh removal. After a median follow-up of 37 (range, 2–91) months there were no recurrences and none of the patients had chronic groin pain.

Conclusions: Mesh removal does not appear to lead to hernia recurrence, suggesting that the strength of a mesh repair lies in the fibrous reaction evoked within the transversalis fascia by the prosthetic material rather than to the physical presence of the mesh itself. It is also possible that deep infection occurring in a mesh enhances this reaction and in fact strengthens the fascia.

Poster Presentation at the Association of Clinical Biochemists (FOCUS 2004)
(Birmingham)
Abstract Published in Proc ACB National Meeting 2004; 49

A SIMPLE METHOD FOR THE ANALYSIS OF URINARY SUCRALOSE IN THE INVESTIGATION OF INTESTINAL PERMEABILITY

Anderson ADG, Greenway G, Cawley M, MacFie, J, Poon P

BACKGROUND AND AIM: A novel technique for the site-specific assessment of intestinal barrier function has recently been described in animals. We have modified this technique for use in humans. The resultant "triple sugar" permeability test requires the analysis of urinary sucralose, a recently developed artificial sweetener, following oral administration. The aim of this study was to develop a simple method for urinary sucralose analysis using high pressure liquid chromatography (HPLC).

METHODS: Sample preparation involved addition of 100μL of phenyl-ß-d-glucopyranoside 10mg/ml (internal standard) to 10ml of urine, which was then passed through a 0.45μm syringe filter. Separation was performed using a reverse-phase C18 column. The mobile phase comprised an isocratic water:methanol mix (70:30), at a flow rate of 1ml/min. Injection volume was 100μL. Detection was with a refractive index detector, and integration based upon peak areas. All samples were assayed in duplicate.

Aqueous and urine-based calibration curves were produced, and sixty standards made by adding sucralose (25-100mg/L) to the urine of randomly selected hospital inpatients. The percentage recovery of sucralose in each standard was used to calculate inter-assay variability.

RESULTS: The calibration curve obtained by plotting sucralose concentration against peak area was linear in the concentration range 10 to 500mg/L ($R^2 = 0.99$). Mean recovery of sucralose in 60 urine-based standards was 103.1% (CV 6.07%). The lower limit of detection was 10mg/L.

CONCLUSIONS: The method described represents a simple assay for urinary sucralose which has acceptable accuracy and precision. In combination with standard dual sugar probe techniques the quantification of urinary sucralose excretion may permit the non-invasive assessment of colonic and small-intestinal barrier function in human subjects.

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	Journal Name	Manuscript No.		Author Received:	No. of pages: 7	PE: Bhuvana

Acta Physiol Scand 2004, 181, 1–7

Evaluation of a triple sugar test of colonic permeability in humans

A. D. G. Anderson,[1] P. K. Jain,[1] S. Fleming,[2] P. Poon,[1] C. J. Mitchell[1] and J. MacFie[1]

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2 *Royal Cornwall Hospital, Truro, UK*

Received 6 February 2004, accepted 20 May 2004
Correspondence: J. MacFie, Consultant Surgeon, Scarborough Hospital, Woodlands Drive, Scarborough YO12 6QL, UK.

Abstract

Aim: Conventional dual sugar tests of intestinal permeability assess only the stomach and small intestine. A novel triple sugar method of assessing colonic permeability has recently been described in animals. This utilizes the non-fermented sweetener sucralose, in addition to conventional sugars. It has been postulated that this test enables the simultaneous assessment of small-intestinal and colonic barrier function in humans. The aim of this study was to evaluate the triple sugar test using healthy volunteers and ileostomists.

Methods: Twenty-one healthy volunteers and 18 ileostomists underwent the triple sugar test. After an overnight fast, subjects drank a solution containing lactulose (5 g), rhamnose (1 g) and sucralose (5 g). Urine was collected for 0–5 h and 5–19 h. Urinary sugars were quantified using HPLC, and 5 and 24-h excretion calculated. Nineteen control subjects and 16 ileostomists also underwent a ^{51}Cr-EDTA permeability test. Permeability data were presented as medians (IQR), and differences between groups analysed with Mann–Whitney *U*-tests.

Results: Lactulose excretion and the 5-h lactulose/rhamnose (L/R) ratio were similar in controls and ileostomists [L/R ratio 0.024 (0.022–0.034) vs. 0.025 (0.022–0.035), $P = 0.210$]. Twenty-four hours excretion of sucralose was significantly higher in control subjects compared with ileostomists [1.41% (1.17–1.68) vs. 0.96% (0.64–1.2), $P = 0.003$]. The same pattern was seen with ^{51}Cr-EDTA [2.73% (2.06–3.76) vs. 2.06% (1.55–2.71), $P = 0.037$] and with lactulose [0.52% (0.42–0.60) vs. 0.25% (0.16–0.35), $P = 0.002$].

Conclusions: Both sucralose and ^{51}Cr-EDTA underwent significant colonic absorption. A significant amount of lactulose also appeared to be absorbed in the colon. This unexpected finding requires further study.

Keywords intestinal permeability, lactulose, rhamnose, sucralose.

The basic premise behind modern tests of intestinal permeability is that the urinary excretion of an orally administered test substance (probe) reflects the non-mediated diffusion of that probe across the intestinal epithelium. Many substances have been used as permeability probes including monosaccharides, oligosaccharides,^{51}chromium-labelled ethylenediaminetetraacetic acid (^{51}Cr EDTA) and polyethylene glycol (PEG). Common practice is to use a combination of a larger molecular weight probe such as lactulose with a smaller probe such as L-rhamnose or mannitol. ^{51}Cr-EDTA has the disadvantage of being a radioisotope, whilst the route of absorption of PEG has not been well defined (Travis & Menzies 1992). The commonly used permeability probes lactulose and ^{51}Cr-EDTA are thought to cross the intestinal epithelium via a para-cellular route (Travis & Menzies 1992). Absorption in healthy volunteers is minimal (<3% of an orally administered dose over 24 h), but this increases in the presence of intestinal disease.

Lactulose is a disaccharide of molecular weight 342.3 Da (Budavari *et al.* 1989). It is not hydrolysed by intestinal enzymes (Bjarnason *et al.* 1995), and undergoes almost 100% urinary excretion over 24 h when administered intravenously (Maxton *et al.* 1986, Elia *et al.* 1987). These properties make lactulose a useful probe for the investigation of intestinal permeability. However, lactulose is a substrate for fermentation by colonic bacteria and is degraded on incubation with stool (Meddings & Gibbons 1998). It is therefore of little use in assessing colonic barrier function, and is regarded as a marker of small intestinal permeability (Travis & Menzies 1992, Bjarnason *et al.* 1995).

Meddings and Gibbons have recently described the use of a new artificial sweetener sucralose (a chlorinated derivative of sucrose) as an intestinal permeability probe in rats (Meddings & Gibbons 1998). Sucralose has a molecular weight similar to that of lactulose [397.6 Da (Budavari *et al.* 1989)]. The authors demonstrated that sucralose was resistant to bacterial fermentation and could be found in high concentrations in the colon following oral administration. This was in marked contrast to lactulose and mannitol, which were detectable only in the stomach and small intestine. The authors concluded that sucralose might be a useful marker of whole-gut permeability. In the situation of raised sucralose excretion with a normal lactulose/mannitol ratio, colonic damage could be inferred. Further, if 24-h lactulose excretion (postulated to represent small intestinal permeability) was subtracted from 24-h sucralose excretion (postulated to represent whole-gut permeability), an islolated measure of colonic permeability might be obtained. This 'triple-sugar' technique has subsequently been used in humans to assess gastrointestinal damage caused by non-steroidal anti-inflammatory drugs (NSAIDs) (Smecuol *et al.* 2001) and nicotine patches (Suenaert *et al.* 2003). These authors used lactulose, mannitol and sucralose as sugar probes.

The aim of this study was to evaluate the triple sugar test as a measure of colonic permeability in healthy volunteers and ileostomists. Sucralose absorption was compared with that of ^{51}Cr-EDTA, an established permeability probe which also resists bacterial degradation and which has previously been used as a measure of colonic permeability. L-rhamnose was used in place of mannitol due to greater experience with this sugar probe in the author's institution (O'Boyle *et al.* 1998, McNaught *et al.* 2002).

Materials and methods

Control subjects

Twenty-one healthy volunteers (12 females) without previous gastrointestinal disease or renal impairment completed the triple sugar test. The median age was 46 (IQR 33-51) years. All subjects were offered the ^{51}Cr-EDTA test within 2 weeks of the sugar test. One subject refused, and one subject failed to complete a 24-h collection, leaving 19 patients (11 females) with ^{51}Cr-EDTA data. One control subject was receiving long-term hormone replacement therapy; none of the other subjects required regular medication. No subject had received NSAIDs within 48 h or alcohol within 24 h of the permeability test.

Six of the control subjects (two females) subsequently underwent the lactulose–hydrogen breath test. No subject had received antibiotic therapy in the month prior to the test.

Ileostomists

Eighteen subjects (eight females) with ileostomies underwent the triple sugar test. Ten subjects had end-ileostomies following colectomy for ulcerative colitis, whilst the remaining eight subjects had loop ileostomies following excision of rectal cancer. There was no evidence of residual disease in any subject. Two subjects declined the ^{51}Cr-EDTA test; the remaining 16 ileostomists (six females) underwent this test within 2 weeks of the sugar test.

The median age of ileostomists was 66 years (IQR 56–70), which was significantly older than control subjects ($P = 0.018$, Mann–Whitney U-test). The median time between formation of ileostomy and the triple sugar test was 20 months (IQR 6–69).

Six ileostomists did not take medication of any description. Of the remaining 12, one took a NSAID (stopped for 48 h prior to the test), three took diuretics, one took an aminosalicylate and one took oral steroids. No subject had received NSAIDs within 48 h of permeability testing, or alcohol within 24 h.

Six of the 18 ileostomists (four females) subsequently underwent a lactulose–hydrogen breath test. No subject undergoing the breath test had received antibiotic therapy in the preceding month.

Ethical approval

The Scarborough Local Research Ethics Committee approved this study. Informed written consent was obtained from every participant. A license for the use of ^{51}Cr-EDTA was obtained from the Administration of Radioactive Substances Advisory Committee.

Administration of the triple sugar test

Subjects were asked to refrain from alcohol for 24 h prior to the test, and to fast from midnight. The triple sugar test was administered between 08.30 and

09.30 hours the following morning. As concentrated sucralose is unpalatable, subjects were asked to quickly drink 30 mL water containing 5 g sucralose (MW 397.6 Da, obtained as analytical grade micronized powder, McNeil Nutritionals, NJ, USA), immediately followed by 120 mL water containing 5 g lactulose (MW 342.3 Da, obtained as 7.5 mL of Duphalac® syrup, Solvay Pharmaceuticals, Marietta, GA, USA) and 1 g L-rhamnose (MW 182.2 Da, obtained as analytical grade powder, BDH Laboratory Supplies, Poole, UK). The combined osmolality of the test solution was 250 mOsmol kg^{-1} (Advanced™ Micro-Osmometer, Model 3MO Plus; Vitech Scientific, Partridge Green, West Sussex, UK).

After ingesting the test solution subjects were allowed to drink water for the first 5 h and to eat and drink freely following this. Subjects were asked to refrain from alcohol until they had completed the 24-h urine collection. Urine passed during the first 5 and last 19 h of the collection period was collected separately into two containers with 1 mL 10% sodium merthiolate as preservative.

Administration of the ^{51}Cr-EDTA test

Pre-test dietary restrictions were as for the triple-sugar test. The test drink contained approximately 1.85 MBq ^{51}Cr-EDTA (MW 340 Da; obtained from Nycomed Amersham, Buckinghamshire, UK) in 120 mL water. Subjects were allowed to drink water for the first 2 h and to eat and drink freely following this. All urine passed during the ensuing 24 h was collected into a container with 1 mL 10% sodium merthiolate as preservative. The osmolarity of the test solution was 7 mOsmol kg^{-1} (Advanced™ Micro-Osmometer, Model 3MO Plus; Vitech).

Analysis of urinary sugars

Urinary lactulose and L-rhamnose concentrations were assayed using HPLC with pulsed amperometric detection, as previously described (Fleming *et al.* 1990, 1993). Sucralose concentrations were assayed using HPLC with refractive index detection (Gilson 133; Gilson, Middleton, USA). Urine samples were prepared by adding 100 μL of internal standard (analytical-grade phenyl-β-D-glucopyranoside, 10 mg mL^{-1}, Fluka) to 10 mL of urine, which was then passed through a 0.45 μm syringe filter (Alltech Associates, Carnforth, UK). Separation was performed on a reverse-phase C18 column [Luna C18(2), 250 × 4.6 mm, particle size 5 μm; Phenomenex Torrance, CA, USA]. An isocratic mobile phase of 30% methanol (Fisher, UK) in water was used at a flow rate of 1 mL min^{-1}. Injection volume was 100 μL. Data from the detector was routed to a data collection unit (PL-DCU; Polymer Laboratories, Amherst, MA, USA) and integration performed using Polymer Laboratories software PL LC/GC Version 2.0. Quantification was based upon the ratio of sucralose peak area to internal standard peak area.

Analysis of 60 standards of sucralose in urine in the concentration range (25–100 mg L^{-1}) demonstrated a mean analytical recovery of 103.1%, with a between-batch coefficient of variation of 6.07%. Each standard was assayed in duplicate, as were all urine samples.

Analysis of urinary ^{51}Cr-EDTA

Urine was assayed for gamma activity using a Counter Ratemeter MS310 (J & P Engineering, Reading, UK). A 400 mL aliquot was counted, along with an appropriate standard prepared from the same stock solution as the subject's dose. All counting was performed until at least 10 000 counts had been obtained, and fractional excretion calculated from the ratio of urine count rate to standard count rate, after a correction had been made for background activity. The between-batch coefficient of variation was 4.54%.

Lactulose–hydrogen breath test

As bacterial overgrowth in the small intestine was a potential confounding factor, six ileostomists and six control subjects also underwent a lactulose–hydrogen breath test, in order to assess the extent of small-intestinal bacterial colonization.

The concentration of end-tidal breath hydrogen was measured using a previously validated technique which employed a hand-held hydrogen meter (Bedfont EC 60 Hydrogen Monitor; Bedfont Technical Instruments, Sittingbourne, UK) (Fleming 1990). A baseline breath hydrogen sample was obtained, and subjects were then asked to drink a solution containing 13.3 g of lactulose (20 mL Duphalac® syrup; Solvay Pharmaceuticals, Marietta, GA, USA) in 120 mL water. Breath hydrogen concentration was measured every 15 min for 3 h, during which time patients were kept 'nil by mouth'. A rise in breath hydrogen concentration of >20 p.p.m. above baseline fasting levels was regarded as significant (Santavirta 1991).

Data collection and statistical analysis

All data was collected by the principal investigator and stored as a series of Microsoft® Excel spreadsheets (Microsoft®, USA). Statistical analyses were performed with XLStatistics (©Rodney Carr 1997–2002) and SPSS® for Windows Version 10.0 (SPSS, Chicago, IL, USA). As the results of permeability testing were not normally distributed, medians with inter-quartile ranges

(IQR) were reported throughout. Differences between groups were compared with the Mann–Whitney *U*-test for continuous variables and the chi-square test for categorical variables.

Results

Permeability tests

The median (IQR) 5-h urine volume for control subjects was 452 (215–708) mL, and for ileostomists 163 (103–297) mL ($P = 0.001$, Mann–Whitney *U*-test). The median 24-h urine volumes for control subjects was 1984 (1400–2504) mL, and for ileostomists 997 (683–1535) mL ($P = 0.007$, Mann–Whitney *U*-test).

Figure 1 demonstrates 5 and 24-h lactulose/rhamnose (L/R) excretion ratios. The 5-h L/R ratio was similar in controls and ileostomists [0.024 (0.022–0.034) vs. 0.025 (0.022–0.035) respectively, $P = 0.210$]. However, the 24-h L/R ratio was significantly higher in controls than ileostomists. It can be seen from Figure 2 that this was due to a twofold difference in lactulose absorption between control subjects and ileostomists.

Figure 3 demonstrates urinary sucralose excretion. Sucralose excretion was significantly lower in ileostomists than in control subjects over both 5 and 24-h; the difference was more marked over 24-h.

Figure 4 demonstrates 24-h urinary ^{51}Cr-EDTA excretion. This was significantly reduced in ileostomists compared with control subjects, in keeping with sucralose and lactulose excretion. ^{51}Cr-EDTA excretion demonstrated a positive correlation with sucralose excretion, however this was not statistically significant ($R = 0.264$, $R^2 = 0.070$; $P = 0.062$).

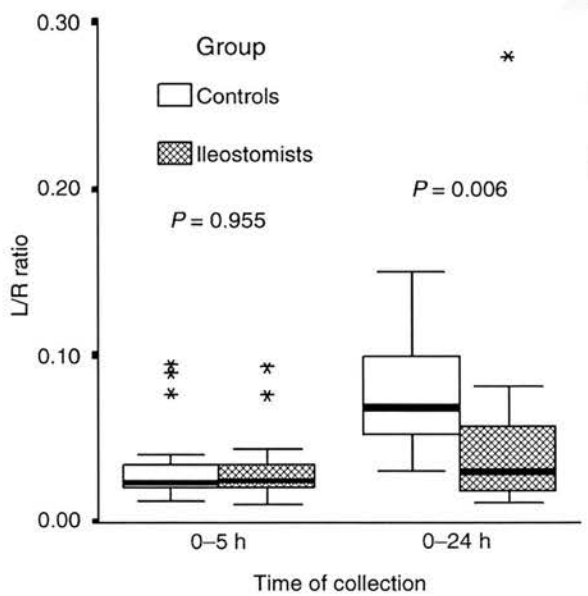

Figure 1 Five and 24-h lactulose/rhamnose (L/R) excretion ratios in control subjects and ileostomists. *P*-values represent Mann–Whitney *U*-test between groups. Bar represents median, box represents interquartile range, error bars represent 'trimmed' range, circles represents 'outliers' (data points with a value between 1.5 and three box lengths from the nearest quartile), asterisks represents 'extremes' (data points with a value greater than three box lengths from the nearest quartile).

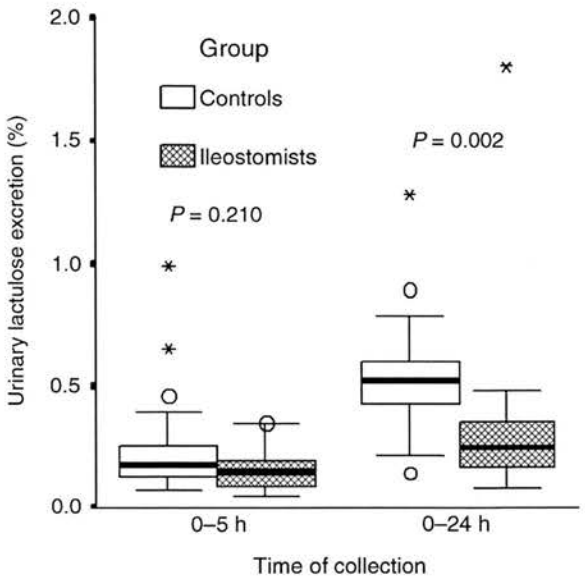

Figure 2 Five and 24-h lactulose excretion in control subjects and ileostomists. *P*-values represent Mann–Whitney *U*-test between groups, symbols are as for Figure 1.

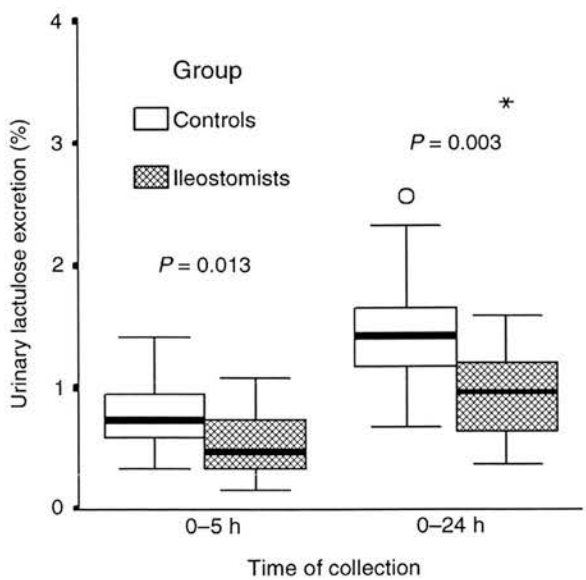

Figure 3 Five and 24-h sucralose excretion in control subjects and ileostomists. *P*-values represent Mann–Whitney *U*-test between groups, symbols are as for Figure 1.

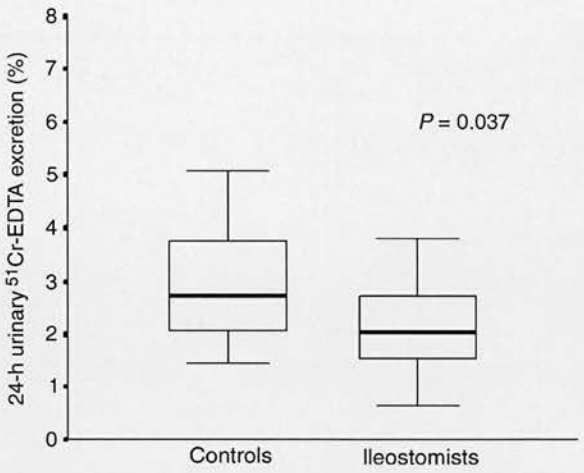

Figure 4 Twenty-four hour excretion of ^{51}Cr-EDTA in control subjects and ileostomists. *P*-values represent Mann–Whitney *U*-test between groups, symbols are as for Figure 1.

The proportion of 24-h probe excretion that occurred in the first 5 h of collection is shown in Table 1.

Lactulose–hydrogen breath test

All six control subjects demonstrated a significant and sustained rise in breath hydrogen excretion following the administration of lactulose (median rise 38 p.p.m. above baseline, range 21–89 p.p.m. above baseline). In contrast, none of the ileostomists demonstrated a significant rise ($P = 0.002$; Fisher's Exact Test). Median oro-caecal transit time (defined as the time breath hydrogen first reached >20 p.p.m. above baseline) was 75 (range 60–165) min in control subjects.

Discussion

The aim of this study was to evaluate a triple sugar test as a measure of colonic permeability in healthy volunteers and ileostomists. The sugars lactulose and rhamnose were used to assess small intestinal permeability (O'Boyle *et al.* 1998, McNaught *et al.* 2002). Sucralose absorption was compared with that of ^{51}Cr-EDTA, an established non-fermented permeability probe. All permeability tests were administered in a standardized manner, and urinary excretion quantified by accurate and precise analytical techniques.

Table 1 Percentage of 24-h probe excretion which occurred in the first 5 h

	Controls (%)	Ileostomists (%)	*P*-value
Lactulose	40.2 (25.5–50.8)	56.9 (27.6–69.4)	0.311
Rhamnose	86.2 (82.3–96.2)	63.5 (54.2–75.2)	<0.001
Sucralose	53.0 (46.6–59.3)	57.5 (38.3–66.0)	0.571

Lactulose/rhamnose excretion ratios and ^{51}Cr-EDTA excretion in control subjects were in keeping with the results of previous studies (Aabakken 1989, Menzies *et al.* 1990, Johnston *et al.* 1996). Sucralose excretion in control subjects was slightly lower than that seen previously (Smecuol *et al.* 2001, Suenaert *et al.* 2003). This could be accounted for by the fact that previous investigators used a hyperosmolar test solution, whereas an iso-osmolar solution was used in the current study. The results of triple sugar testing confirmed that lactulose and sucralose are absorbed in minimal amounts in healthy subjects.

There was no difference in small intestinal permeability between control subjects and ileostomists, as assessed by a 5-h L/R excretion ratio (Fig. 1). Control subjects, however, exhibited significantly greater 24-h excretion of both lactulose and sucralose. Both sugar probes behaved in a similar manner to that of ^{51}Cr-EDTA. The most obvious explanation for the increased 24-h excretion of sucralose and lactulose in controls is that the difference represented colonic absorption, a finding which has been demonstrated previously for ^{51}Cr-EDTA (Elia *et al.* 1987, Jenkins *et al.* 1991). This presupposes that small intestinal absorption was similar in both groups.

It could, however, be argued that the remaining small intestine was not 'normal' in the ileostomists, due to residual disease, alteration in absorptive function, abnormal transit time, or bacterial overgrowth. The authors consider each of these possibilities unlikely, for the following reasons. The 18 ileostomists were selected on the basis of an underlying diagnosis of ulcerative colitis or rectal cancer, with normal small bowel. No subject had clinical evidence of gastrointestinal disease at the time of study. Furthermore, 5-h lactulose and L/R excretion ratios, the traditional measures of small intestinal permeability, were similar between ileostomists and controls. This suggests that small intestinal paracellular permeability and transit were not significantly affected by the formation of an ileostomy, a finding which is in keeping with published data (Elia *et al.* 1987, Jenkins *et al.* 1991). The reduced urine volumes in ileostomists did not affect absolute or fractional 5-h lactulose excretion, suggesting that a difference in renal blood flow between the two groups was not a significant confounding factor. Due to practical considerations, the lactulose–hydrogen breath test was performed in a random selection of six ileostomists and six control subjects. This test provided a direct measure of bacterial fermentation in the intestinal tract. In the sample studied, no ileostomist demonstrated measurable fermentation of lactulose, suggesting that bacterial colonization of the small intestine did not occur to a significant degree.

We conclude, therefore, that the small intestine of ilestomists and control subjects was similar in terms of transit, permeability and bacterial load. Differences in 24-h permeability between the two groups were therefore likely to be a consequence of colonic permeability.

Estimation of colonic absorption of sucralose

The 24-h excretion of sucralose was higher in controls than ileostomists, by a factor of 1.48. Assuming that small intestinal sucralose absorption was similar in ileostomists and controls, the amount of sucralose absorbed in the colon of control subjects could be approximated by subtracting the 24-h sucralose excretion in ileostomists (median 0.955%) from the 24-h excretion in controls (median 1.41%). This gives a hypothetical colonic absorption of 0.455%. This figure represents 32% of the 24-h sucralose excretion in control subjects, implying that approximately a third of sucralose absorption occurred in the colon.

As neither sucralose nor ^{51}Cr-EDTA is a substrate for colonic bacterial fermentation, it is not surprising that a significant proportion of 24-h urinary excretion resulted from colonic permeation. Indeed, this has been the premise for using these probes to measure colonic permeability in conditions such as inflammatory bowel disease (Jenkins *et al.* 1988), NSAID-induced intestinal damage (Smecuol *et al.* 2001), and following the application of nicotine patches (Jenkins *et al.* 1991). However, as both probes are absorbed throughout the length of the gastrointestinal tract, it is impossible to differentiate small intestinal from colonic permeation when interpreting a single 24-h urinary excretion figure. Rather, 24-h ^{51}Cr-EDTA or sucralose excretion represents 'whole gut' permeability.

Estimation of colonic absorption of lactulose

Five and 24-h lactulose excretion is shown in Figure 2. Lactulose excretion over 24-h in control subjects (median 0.517%) was greater than that in ileostomists (median 0.248%), by a factor of 2.08. This finding is at odds with the results of previous studies in a small number of ileostomists, which demonstrated no difference in 24-h lactulose excretion between ileostomists and control subjects (Elia *et al.* 1987, Jenkins *et al.* 1991).

In the absence of a difference in small intestinal permeability, it can be assumed that the difference in 24-h excretion between control subjects and ileostomists (0.269%) was a consequence of colonic absorption. The results of this study imply that approximately 50% of lactulose absorption occurred in the colon of control subjects, following an oral dose of 5 g. This figure might be expected to vary depending on the load presented to the colon, and the capacity of colonic bacteria to ferment lactulose.

This unexpected finding of colonic lactulose absorption has important implications for the use of dual and triple sugar permeability tests. In particular, the subtraction of 24-h lactulose absorption from that of a non-fermented probe (such as sucralose) is unlikely to result in a meaningful estimation of colonic permeability, as lactulose itself appears to be absorbed in the colon. Further investigation into the site of lactulose absorption is warranted.

Time-course of probe excretion

Table 1 demonstrates the proportion of 24-h probe excretion that occurred in the first 5 h, for each of the three sugar probes. It can be seen that, in the ileostomists, almost as much sucralose was excreted in the latter part of the collection (5–24 h) as was excreted in the first 5 h. As small-intestinal transit time is considerably less than 5 h in the vast majority of subjects (Ladas *et al.* 1989, Jorge *et al.* 1994), this delay in excretion is likely to represent the lag between absorption from the intestinal lumen and renal excretion. Although studies of renal sucralose excretion have not yet been performed in humans, studies using lactulose have demonstrated a significant delay between intravenous administration and urinary excretion (Maxton *et al.* 1986, Elia *et al.* 1987).

This finding casts doubt upon the practice of using 'delayed' probe excretion as a measure of colonic permeability. In a recent study investigating the effect of nicotine patches and NSAIDs on the gastrointestinal tract, Suenaert *et al.* (2003) reported 6–24 h urinary sucralose excretion as being synonymous with colonic permeability. However, the results of the current study indicate that a proportion of 6–24 h sucralose excretion may have originated from small intestinal, rather than colonic, absorption.

Summary

The results of this study confirm sucralose and ^{51}Cr-EDTA to be markers of 'whole-intestinal' permeability. It is estimated that between a quarter and a third of 24-h absorption occurs in the colon. Regrettably, the triple sugar test cannot be used to give an isolated measure of colonic permeability, due to the confounding factor of colonic lactulose absorption. However, in the situation of a normal 5-h L/R ratio, an increase in 24-h sucralose excretion is likely to be indicative of raised colonic permeability.

The results of this study also suggest that a significant amount of lactulose is absorbed in the colon. The results of previous studies involving lactulose/mannitol or L/R

tests may need to be re-interpreted in the light of this unexpected finding.

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